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LOYOLA UNIVERSITY CHICAGO

PROTECTION FROM *STAPHYLOCOCCUS AUREUS* BLOODSTREAM INFECTION BY  
PROBIOTIC EXOPOLYSACCHARIDE

A DISSERTATION SUBMITTED TO  
THE FACULTY OF THE GRADUATE SCHOOL  
IN CANDIDACY FOR THE DEGREE OF  
DOCTOR OF PHILOSOPHY

PROGRAM IN MICROBIOLOGY AND IMMUNOLOGY

BY  
WONBEOM PAIK  
CHICAGO, IL  
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## LIST OF ABBREVIATIONS

AdsA	Staphylococcus aureus adenosine synthase A
Agr	Staphylococcus aureus accessory gene regulator
AID	Activation-induced cytidine deaminase
Akt	Protein kinase B
ALDH	Aldehyde dehydrogenase
aM	Attomolar
AMP	Antimicrobial peptide
APC	Allophycocyanin
Arg-1	Arginase 1
BMDC	Bone marrow-derived dendritic cell
BM-PMN	Bone marrow neutrophils
BV	Brilliant Violet
C5aR	Complement C5a receptor
CA-MRSA	Community-acquired methicillin-resistant Staphylococcus aureus
CBA	Cytometric bead array
CCL2	C-C motif chemokine ligand 2
CCR5	C-C motif chemokine receptor 5
CD	Cluster of differentiation
CDI	Clostridium difficile infection
CFU	Colony forming unit

CHIPS	Chemotaxis inhibitory protein of <i>Staphylococcus aureus</i>
CRAMP	Cathelicidin-related antimicrobial peptide
CTV	CellTrace Violet
CX <sub>3</sub> CR <sub>1</sub>	C-X <sub>3</sub> -C motif chemokine receptor 1
CXCL <sub>1</sub>	C-X-C motif chemokine ligand 1
CXCR <sub>2</sub>	C-X-C motif chemokine receptor 2
Cy7	Cyanine 7
d.p.i.	Days post infection
DC	Dendritic cell
DEAE	Diethylaminoethyl
DIC	Disseminated intravascular coagulation
DNA	Deoxyribonucleic acid
DPI	Diphenyleneiodonium
DSS	Dextran sodium sulfate
E2-PDH	E2 subunit of pyruvate dehydrogenase complex
EIEC	Enteroinvasive <i>Escherichia coli</i>
EPEC	Enteropathogenic <i>Escherichia coli</i>
EPS	<i>Bacillus subtilis</i> exopolysaccharide
ERK <sub>1</sub>	Extracellular signal-regulated kinase 1
FADD	Fas-associated death domain
FBS	Fetal bovine serum
Fc	Fragment crystallizable region
FITC	Fluorescein isothiocyanate

Foxp3	Forkhead box protein P3
GALT	Gut-associated lymphoid tissue
GATA-6	GATA-binding factor 6
G-CSF	Granulocyte colony stimulating factor
GI	Gastrointestinal
GM-CSF	Granulocyte-macrophage colony stimulating factor
GPCR	G protein-coupled receptor
GPI	Glycosylphosphatidylinositol
H&E	Hematoxylin and eosin
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
i.p.	Intraperitoneal
IBD	Inflammatory bowel disease
ICAM-1	Intercellular adhesion molecule 1
IFN- $\gamma$	Interferon gamma
IFN- $\gamma$ R1	Interferon gamma receptor 1
IL-10	Interleukin 10
iNKT cell	Invariant natural killer T cell
iNOS	Inducible nitric oxide synthase
IPEX	Immune dysregulation, polyendocrinopathy, X-linked syndrome
IPTG	Isopropyl $\beta$ -D-1-thiogalactopyranoside
iTreg cell	Inducible regulatory T cells
IV	Intravenous
JAK	Janus kinase

KC	Keratinocyte-derived chemokine
kDa	Kilodalton
KO	Knockout
LAC	Los Angeles county
LB	Luria-Bertani medium
LPM	Large peritoneal macrophage
LPS	Lipopolysaccharide
LukED	Leucocidin ED
M cell	Microfold cell
MAP	Mean arterial pressure
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein 1
MD-2	Myeloid differentiation factor 2
MEM	Minimal essential medium
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MIP-1 $\alpha$	Macrophage inflammatory protein 1 alpha
mLN	Mesenteric lymph node
mmHg	Millimeter mercury
MMP	Matrix metalloproteinase
MNP	Mononuclear phagocyte
mRNA	Messenger ribonucleic acid
MRSA	Methicillin-resistant Staphylococcus aureus
Msgg	Minimal salts glutamate glycerol



mtROS	Mitochondrial reactive oxygen species
Muc2	Mucin 2
MyD88	Myeloid differentiation primary response protein 88
MΦ	Macrophage
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NaHCO <sub>3</sub>	Sodium bicarbonate
NETosis	Neutrophil extracellular trap-forming cell death
NF-κB	Nuclear factor kappa light chain enhancer for activated B cells
NK cell	Natural killer cell
NO	Nitric oxide
nTreg cell	Natural regulatory T cells
Nuc	Staphylococcus aureus nuclease
OD	Optical density
OVA	Ovalbumin
PBS	Phosphate-buffered saline solution
PD-L1	Programmed death-ligand 1
PE	Phycoerythrin
pIgR	Polymeric immunoglobulin receptor
PMA	Phorbol 12-myristate 13-acetate
PNPP	para-nitrophenyl phosphate
PRR	Pattern recognition receptor
PSA	Bacteroides fragilis polysaccharide A
PVL	Panton-Valentine leucocidin

qSOFA	Quick sepsis-related organ failure assessment score
R-848	Resiquimod
RA	Retinoic acid
RANTES	Regulated on activation, normal T cell expressed and secreted protein
RAW-SEAP	RAW 264.7 cells with NF- $\kappa$ B/AP-1 SEAP reporter
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rpm	Rounds per minute
RPMI	Modified Roswell Park Memorial Institute medium
SAg	Superantigen
SCFA	Short chain fatty acid
SCIN	Staphylococcal complement inhibitor
SD	Standard deviation
SE	Staphylococcal enterotoxin
SEAP	Secreted embryonic alkaline phosphatase
SEI	Staphylococcal enterotoxin-like
SHM	Somatic hypermutation
sIgA	Secretory immunoglobulin A
SodA	Superoxide dismutase A
SOFA	Sepsis-related organ failure assessment score
SPF	Specific pathogen-free
SPM	Small peritoneal macrophage
SSL3	Staphylococcal superantigen-like protein 3
SSTI	Skin and soft tissue infection

STAT	Signal transducer and activator of transcription
TCR	T cell receptor
TER	Transepithelial resistance
TFH cell	T follicular helper cell
TFR cell	T follicular regulatory cell
TGF- $\beta$	Transforming growth factor beta
TIR	Toll-interleukin-1 receptor homology domain
TLR2	Toll-like receptor 2
Tn	Transposon
TNFR1	Tumor necrosis factor receptor 1
TNF- $\alpha$	Tumor necrosis factor alpha
Tr1 cell	Type I regulatory T cell
TRADD	Tumor necrosis factor receptor type 1-associated death domain protein
TRAF2	Tumor necrosis factor receptor-associated factor 2
Treg cell	Regulatory T cell
TRIF	Toll-interleukin-1 receptor domain-containing adaptor-inducing interferon beta
TSA	Tryptic soy agar
TSB	Tryptic soy broth
V gene	Immunoglobulin variable gene
VCAM-1	Vascular cell adhesion molecule 1
WT	Wild-type
ZO-1	Zonula occludens protein 1
$\gamma$ -PGA	Gamma-polyglutamic acid

$\Delta$ EPS      Exopolysaccharide preparation from *Bacillus subtilis* DS5187 (epsH mutant)

## ABSTRACT

*Staphylococcus aureus* is known to cause severe systemic infection with high mortality rates. Antibiotics are the only approved therapy for patients, but the widespread prevalence of antibiotic-resistant strains limits treatment efficacy, and many patients succumb to the disease. Many probiotic agents are commercially available, but the mechanisms by which they benefit are not known. Understanding these mechanisms will help develop novel therapeutics that can improve healthcare, including systemic infections by *S. aureus*.

*Bacillus subtilis* is a probiotic bacterium that produces an exopolysaccharide (EPS) that induces anti-inflammatory macrophages (M $\Phi$ ). We tested if EPS could be used for systemic *S. aureus* infections in mice and found that EPS-treated mice had improved outcomes, reducing bacterial load and inflammation. We tested for the mechanism behind this protection and found that EPS-induced M $\Phi$ , while they resemble anti-inflammatory M2 M $\Phi$ , restrict the growth of internalized *S. aureus* through reactive oxygen species like that of pro-inflammatory M1 M $\Phi$ . In addition, EPS-induced M $\Phi$  retained their anti-inflammatory capacity to limit T cell activation in response to *S. aureus* superantigens, suggesting that EPS-induces a “hybrid” M $\Phi$  population with both M1- and M2-like qualities, limiting *S. aureus* growth. *In vivo*, we found that *S. aureus* induces interferon gamma (IFN- $\gamma$ ) production through natural killer (NK) cells, a way to manipulate the host immune system for its own pathogenesis. Furthermore, EPS-

treated mice did not make IFN- $\gamma$  in response to *S. aureus*, suggesting that EPS protects hosts from *S. aureus* also by limiting IFN- $\gamma$  production.

We suggest that EPS primes the host immune system to bolster its antimicrobial activity and also limit inflammation, which prevents *S. aureus* from manipulating the host response for its own pathogenesis, leading to reduced disease burden and improving outcomes. By our understanding of EPS mechanism for protection, we have identified a potential strategy to help patients with systemic *S. aureus* infection.

CHAPTER ONE  
REVIEW OF LITERATURE  
**A Brief History of Probiotics**

Human beings come in contact with microbes ubiquitously throughout their daily lives. A common way by which people encounter microbes is through consumption of fermented food products, depicted even in ancient Egyptian hieroglyphs. Many cultures have adopted fermented food products in their diet, probably due to fermentation being used as a method for food storage. Today, a variety of different fermented food products are readily available due to extensive globalization. In addition, recent advances in our knowledge of the microbes that mediate fermentation have pointed to the potential health benefits of fermented food products, and with the emergence of the wellness industry, microbial encounter through consumption of fermented food products is more widespread than ever.

While the story of fermentation through microbes originates from early human history, the microbes that mediate host benefits were not appreciated until more recently. The first probiotic, microbes that benefit the host, was not conceptualized until Élie Metchnikoff associated lactic acid-producing bacteria, termed Bulgarian bacillus at the time, in yogurt with longevity of life in 1908 (Metchnikoff, 1908). The first accepted trial with probiotics was reported in 1922 when Leo Frederick Rettger and Harry A. Cheplin observed apparent improvements in chronic constipation, diarrhea, and eczema in 30 patients through administration of *Lactobacillus acidophilus*

(Rettger and Cheplin, 1921), though it should be noted that randomized clinical trials using placebos were not standard until about the 1950s. Since then, the number of publications and randomized clinical trials related to probiotics has surged, especially since the 2000s (McFarland, 2015). Today, a variety of probiotic products are readily available to the public commercially, behind an industry with an estimated value of \$87 billion (Stanton et al., 2001). Despite widespread use for apparent health benefits, the specific mechanisms that underlie these benefits are generally not clear, limiting the use of probiotics in mainstream healthcare settings since measuring efficacy and predicting potential adverse effects are difficult. Therefore, understanding the underlying biology of probiotics and their interactions with the host is critical for transforming beneficial microbes into a novel sector of healthcare innovations.

### **Probiotic Mechanism of Action: Colonization Resistance**

#### **Microbial Colonization of the Gastrointestinal Tract**

Most studies of probiotics and their mechanisms of action focus on the gastrointestinal (GI) tract, as many probiotic organisms have been isolated from the GI tract and are administered orally such as Henry Tissier's *Bifidobacterium bifidum*, Alfred Nissle's *Escherichia coli* Nissle 1917, and Minoru Shirota's *Lactobacillus casei* subspecies *Shirota*. The GI tract hosts a significant number of microbial cells, a number estimated to equal the number of human cells in the entire body (Sender et al., 2016). However, different sections within the GI tract provide diverse environmental conditions for which microbes must adapt. In the oropharyngeal lumen, salivary secretions contain a variety of digestive enzymes that dictate the kinds of nutrients that are available. In addition, saliva contains antimicrobial molecules such as lysozyme and peroxide, dictating what kind of microbes can survive within the oropharyngeal mucosa.



In the gastric tissue, parietal cells produce hydrochloric acid that lowers luminal pH to 1, a major adaptive barrier for microbes. In fact, a major quality of what is considered a “good” probiotic is the ability to survive passage through the acidic gastric lumen, so they could efficiently colonize the lower GI tract (Hill et al., 2014). The small intestine is subject to many digestive enzymes and molecules such as pancreatic secretions, bile secretions, and brush border enzymes intrinsic to the small intestines. Like in the oropharynx, these enzymes dictate the types of nutrients available and subsequently put selective pressure on microbes to adapt. In addition, bile acids can have antimicrobial activities (Binder et al., 1975), which put additional selective pressure on microbes. Also, the lower GI tract is under massive immune surveillance that the microbes must deal with to avoid elimination from the GI mucosa. As for the colon, a key distinguishing environmental feature is the low oxygen content, measured at below 1 millimeter mercury (mmHg) (Albenberg et al., 2014). Consequently, anaerobic bacteria are enriched in the microbiome of the colon (Hugon et al., 2015; The Human Microbiome Project Consortium, 2012). While the lower GI tract is the most densely colonized, it should be noted that microbes are not limited to the GI tract. In fact, the microbiome exists within other organs as well, including skin, upper and lower respiratory tracts, and genitourinary tract, each with its own environmental factors that require their own specific adaptations for microbial survival (The Human Microbiome Project Consortium, 2012). The diversity of the microbiota is further enhanced because each organ harbors specialized spaces with distinct environments. While one would typically assume an oxygen-rich environment in the oropharyngeal mucosa as it shares space with the upper airways, certain places such as the deep crypts of tonsils harbor a robust community of anaerobic microbes (Brook, 1981), indicating an anaerobic microenvironment within the

tissue. In the gastric tissue, the lumen generally measures at low pH as discussed above, but it is thought that the pH may be less acidic in spaces juxtaposed to the gastric epithelium due to bicarbonate secretion by the non-parietal cells, assumed to be a mechanism to protect gastric epithelium from acidic damage (Flemström, 1977). This creates a microenvironment distinct from the lumen of the gastric tissue, where the pH is relatively less acidic and allows for bacteria to survive and colonize. These microenvironments are also subject to modulation by host physiology and behavior since neutralization of gastric acid often occurs in patients given proton pump inhibitor therapy which can cause a  $10^4$ -fold increase in gastric luminal pH with just a single dose (Laine et al., 2008). Therefore, while a vast number of microbes colonize a variety of different parts of the human body, they do so specifically within given microenvironments, termed niches, with specific adaptations to the nutrients and other factors particular to that niche. As such, microbes must compete with other microbes, as well as with the host, for limited space and nutrients to survive. To this end, many probiotics have adapted specific strategies to outcompete other microbes, and through many of these mechanisms, probiotics can limit colonization of host by pathogens. The ability of the host and its microbiota to not allow colonization by pathogens is termed colonization resistance, the very first mechanism by which probiotics were thought to benefit the host as conceptualized by Metchnikoff, to prevent colonization by microbes that promoted putrefaction of the GI tract (Metchnikoff, 1908).

## Direct Competition of Microbes

Today, there are many mechanisms described by which microbes compete against other microbes. *Lactobacilli* are the prototypic probiotic bacterium studied early on in the field, aptly named for their production of lactic acid through their metabolic processes, which gave the sour taste associated with probiotic-containing foods. Lactic acid production reduces pH, as low as pH 4.5 in the female genitourinary tract in humans (Andersch et al., 1986), which limits the growth of other bacteria not adapted for acidic conditions; presence of *Lactobacilli* and low pH in the female genitourinary tract was associated with reduced risk for bacterial vaginosis (Spiegel et al., 1980). This acid production or “souring” quality in milk was originally the quality associated with the beneficial activity (Rettger and Cheplin, 1921). Still, acid production is not the only mechanism by which *Lactobacillus* species can compete against other bacteria. *Lactobacilli* can use oxygen as the terminal electron acceptor for its metabolism through flavoproteins that catalyze the formation of hydrogen peroxide ( $H_2O_2$ ) that can be toxic to other microbes (Eschenbach et al., 1989). The spent culture medium of *Lactobacilli* was demonstrated to have antibacterial activity on *Staphylococcus aureus in vitro* (Dahiya and Speck, 1968). This effect was ameliorated by treating the spent medium with catalase, an enzyme that converts  $H_2O_2$  to water and oxygen, indicating that  $H_2O_2$  was responsible for the antibacterial activity of *Lactobacilli*. Though the specific molecules and modifications affected by  $H_2O_2$  in target microbes are not known, it is generally thought that the oxidative damage from superoxide radicals derived from the peroxide disrupts cellular processes, leading to bactericidal or bacteriostatic activity (Dahiya and Speck, 1968; Thomas et al., 1994).

*Lactobacilli* also produce other factors that confer a broad antibacterial activity *in vitro*, as *L. acidophilus* LA1 was shown to secrete molecules that inhibit growth of many gram positive and gram negative pathogens such as *Listeria monocytogenes*, *S. aureus*, *Salmonella typhimurium*, and *Pseudomonas aeruginosa*, though the molecules that mediate this antibacterial activity have not been identified (Bernet-Camard et al., 1997).

Within the niche, microbes compete for nutrients against each other and against the host as well. The fiercest competitions occur over trace nutrients such as metals that serve as cofactors in many biological processes. Iron is the best studied of these and is actually one of the most abundant elements on Earth (Morgan and Anders, 1980), though most are inaccessible to biological entities. Many forms of life require relatively high concentrations of iron, but iron is quite insoluble under physiologic conditions. Ferric iron, the free aqueous form, have an estimated solubility at attomolar (aM,  $10^{-18}$ ) concentrations. The host also requires iron for many physiologic processes, and therefore absorbs much of the dietary iron in the proximal small intestine and tightly regulates circulating iron levels to avoid toxicity. Therefore, iron is very limited in the extracellular space, which also serves as a host defense mechanism to limit bacterial overgrowth and virulence, and patients under iron overload conditions often fare worse during infection (Barry and Reeve, 1977). Bacteria have developed many iron acquisition systems designed to scavenge iron from the environment, with the best-characterized system being the siderophores. Siderophores are essentially metal-binding chelators secreted by the bacteria, with an affinity for iron that generally thought to exceed host iron chelators like transferrin (Fetherston et al., 2010). Once the iron-siderophore complex is formed, bacteria take up the iron through active transport using specific

transporter systems (Bäumler et al., 1998). By sequestering the trace iron from the environment, uptake of iron by a probiotic bacterium can contribute to iron limitation within the niche, potentially limiting the growth of pathogens. An example of this is demonstrated in *E. coli* Nissle 1917, a probiotic strain of *E. coli* isolated from a soldier who was thought to be resistant to an outbreak of diarrhea by Alfred Nissle in 1917. Administration of *E. coli* Nissle 1917, but not a mutant defective in iron-acquisition, reduced colonization by the enteric pathogen *S. typhimurium* in a murine model (Deriu et al., 2013), demonstrating an ability of a probiotic to outcompete an already established pathogen through iron sequestration. As discussed above, iron is not the only trace metal for which the bacteria compete. In the case of *Campylobacter jejuni*, a commensal bacterium in the GI tract of chickens, successful colonization depends on its zinc acquisition system in normal chickens but not in germ-free chickens, indicating that *C. jejuni* uses its zinc acquisition system for colonization when zinc is limited due to microbial competition (Gielda and DiRita, 2012). It should be noted that metal acquisition can have effects beyond the context of microbial competition, since in many pathogens the levels of intracellular metals dictate expression of virulence factors (Fetherston et al., 2010; Kirienko et al., 2013; Martin et al., 2013; Peralta et al., 2016). Therefore, one way probiotics could benefit hosts through metal sequestration may also be through suppressing virulence of pathogens by limiting their acquisition of metals.

There are also more direct mechanisms by which bacteria compete with others, such as bacteriocins that directly kill other bacteria. Bacteriocins are a broad group of peptides secreted by bacteria that kills targets but not the producer, due to the presence of immunity proteins. Due to their heterogeneous nature, the mechanisms of action for bacteriocins are quite diverse, though many mechanisms resemble those of current

antimicrobial agents such as inhibition of peptidoglycan synthesis, pore formation, inhibition of deoxyribonucleic acid (DNA) gyrase, and inhibition of protein synthesis (Diep et al., 2007; Kobayashi et al., 2010; Parks et al., 2007; Piper et al., 2009).

*Lactobacillus salivarius* subspecies *salivarius* UCC118 is a probiotic that produces the bacteriocin ABP-118 that has antibacterial activity against *L. monocytogenes in vitro* (Flynn et al., 2002), demonstrating the potential for probiotic bacteriocin-mediated inhibition of a pathogen. Still, the most interesting aspect of bacteriocins is that they are genetically amenable for engineering, due to their proteinaceous nature. This potential is best demonstrated in heterologous expression of ABP-118 in other bacteria such as *Lactobacillus plantarum* and *Lactobacillus lactis* (Flynn et al., 2002), showing that probiotics could be engineered to produce bacteriocins to target specific pathogens. Still, the direct antibacterial activities of bacteriocins could give rise to resistant pathogens through mutations, primarily the same concern with any novel antimicrobial agents. In summary, probiotic bacteria use a variety of mechanisms to compete within the niches, and through these mechanisms, contribute to host resistance to colonization by pathogens.

### **Competition in the Context of the Microbiota**

*Lactobacilli* were the first probiotic embraced by the mainstream scientific community and have been studied for more than a century (Lee et al., 2019; Metchnikoff, 1908; Rettger and Cheplin, 1921). The principal mechanism by which they benefit is thought to be by directly limiting the growth of pathogenic microbes through the ways described above. Under this scenario, orally administered *Lactobacillus* probiotic would survive the harsh conditions of the upper GI tract and colonize the lower GI tract with great efficiency where it would mediate its beneficial effects; this trait of a probiotic is

stressed even in the most recent definition of what constitutes a “good” probiotic (Hill et al., 2014). Initially, *Lactobacilli* were reported to represent the majority of the culturable bacteria from human feces (Rettger and Cheplin, 1921). Of course, anaerobic culture techniques were not developed until the 1960s, and studies after this era demonstrated that orally administered *Lactobacilli* species only form marginal populations within the human GI tract, at best estimated to be 0.01% of culturable bacteria (Dal Bello et al., 2003; Finegold et al., 1974). This observation from multiple studies accounting for anaerobic bacteria seemed inconsistent with the idea that *Lactobacilli* exclude pathogens in the GI tract through direct competition, given that other bacteria made up the dominant population. Since then, many probiotics have been screened for colonization efficiency (Jacobsen et al., 1999). For example, specific strains of *Lactobacillus* probiotics with high colonization efficiency such as *Lactobacillus rhamnosus* GG have been identified, and have been the focus of probiotic studies (Canani et al., 2015; Jacobsen et al., 1999; Yan et al., 2016).

The GI tract harbors a complex community of microbes, collectively termed the microbiota, that in recent years gained appreciation for its ability to affect host physiology in virtually every organ system (Abrams and Bishop, 1967; Atarashi et al., 2011; Furusawa et al., 2013; Mazmanian et al., 2008; Al Nabhani et al., 2019; Sharon et al., 2010; et al., 2015; Tyagi et al., 2018). The underlying mechanisms by which the microbiota affect host physiology are not entirely clear, but it is well accepted that the different composition of the microbiota correlates with the different physiologic conditions or processes, including colonization resistance against pathogens. Likely, host environmental contact plays a great role in the composition of the microbiota, as it would dictate what microbes are introduced into the system. Once exposed, host factors

such as barriers, nutrient availability, already existing microbes within the host, and immunity would select certain microbes for colonization in various niches. Within niches, individual microbial members compete with one another to dictate which would successfully occupy the niche, forming the microbiota. Some members of the microbiota could indirectly affect the microbial composition by affecting host physiology, altering the niche microenvironment. Therefore, a vast number of relatively simple mechanisms from individual microbes combine to form a complex network of interactions that likely dictate the overall composition of the microbiota within the host. In turn, this may suggest that the introduction of a relatively minor member such as a *Lactobacillus* probiotic into the GI tract could have profound downstream effects on pathogen exclusion through the change in microbial composition in response to *Lactobacilli* or other probiotics. Of course, the exact composition of the microbiota responsible for pathogen exclusion is not known, and how probiotics specifically alter the composition of the microbiota has not been extensively studied. For now, there is an interest in using a combination of probiotic organisms to benefit hosts such as VSL#3 (Gionchetti et al., 2000) that is commercially available, with the idea that by providing multiple agents of supposedly “good” bacteria, more beneficial mechanisms could be deployed, and better host colonization resistance against pathogen could be achieved. Understanding the dynamics of microbial composition in the context of host resistance to pathogens will be needed to understand better the full range of ways probiotics could promote exclusion of pathogens.

Ironically, the most successful form of microbial therapy designed to alter the microbial composition is one of the oldest: fecal transplantation. Fecal transplantation involves the transfer of fecal matter and its microbial content from a healthy donor to a



recipient in an attempt to treat disease and is supposedly documented in a 4<sup>th</sup>-century traditional Chinese medicine textbook (Zhang et al., 2012). In the modern era, fecal transplantation is most often used to treat *Clostridium difficile* infection (CDI), a severe diarrheal disease in patients after antibiotic use due to GI dysbiosis. CDI patients are initially treated with antibiotics which is efficacious, but this perpetuates the GI dysbiosis that allows for *C. difficile* colonization in the first place, and many patients experience recurrent infections (McDonald et al., 2018).

In a clinical trial, fecal transplantation was attempted on patients suffering from recurrent CDI with the idea that resolving the dysbiosis by the transfer of a “healthy” microbiota will restore colonization resistance to the pathogen and cure disease. Compared to vancomycin, the standard therapy, fecal transplantation was so much more efficacious that the study, although controversial, was stopped early with the conclusion that fecal transplantation is the superior therapy (Van Nood et al., 2013). Currently, fecal transplantation is recommended for patients with more than two recurrent CDI episodes (McDonald et al., 2018). Still, what constitutes a “healthy” microbiota is unclear, let alone the methods to standardize the criteria for selecting “healthy” donors for obtaining transplant material. In addition, fecal transplantation has been extensively used in animal models to transfer physiologic traits to demonstrate the role of microbiota in host processes, including obesity (Ridaura et al., 2013). Thus, fecal transplantation has the potential to be therapeutically useful in many conditions, but without a complete understanding of how specific microbial compositions affect physiology, unintended consequences such as transfer of metabolic syndromes could occur (Alang and Kelly, 2015). Nevertheless, a key mechanism by which probiotics could benefit the host is through competition for nutrients and niches for survival, which can

directly or indirectly limit colonization of the host by pathogens. Probiotics also possess these mechanisms to outcompete pathogens, and the potential to use microbes directly for therapy is demonstrated in one of the oldest therapies. A better understanding of how members of the microbiota confer host colonization resistance to pathogens will help develop specific microbial therapies that are efficacious and result in adverse effects that are predictable and manageable.

### **Probiotic Mechanism of Action: Manipulation of the Host Barrier The Gastrointestinal Epithelial Barrier, the Host-Microbe Interface**

The key feature of the host immune system is the ability to selectively respond against foreign pathogenic antigens but not innocuous self- or non-self-associated antigens. This challenge is perhaps best represented within host mucosal surfaces, especially the GI tract, where the host is in perpetual contact with the members of the commensal microbiota. Here, mounting an inappropriate response against food or commensal antigens would result in an inflammatory response that could impede normal functions of the GI tract essential for life. Such dysregulation of GI immune homeostasis is thought to cause GI disorders such as inflammatory bowel disease (IBD) and colon cancer (Feng et al., 2015; Al Nabhani et al., 2019; Willing et al., 2009). The immune system harbors a complex array of mechanisms to maintain immune homeostasis, especially at mucosal sites (Chung et al., 2011; Coombes et al., 2007; Craig and Cebra, 1971; Gabanyi et al., 2016; Sakaguchi et al., 1995). However, failure to respond to potentially invasive pathogens can lead to dissemination of the pathogen. Thus, the mucosal immune system must harbor mechanisms to tightly regulate immunity and strike a delicate balance to ensure host survival. This process generally

starts at the point of contact between the host and microbes, the intestinal epithelial barrier.

The GI tract is the most significant barrier surface, lined by a single layer of columnar epithelial cells that are designed to absorb nutrients while restricting translocation of microbes from the lumen. To prevent inappropriate triggering of immune responses against commensals within the lumen, intestinal epithelial cells generally sequester some of their pattern recognition receptors (PRR) to surfaces in deep crypts or on the basolateral side of the cell, especially in the colon where microbes most heavily colonize (Chabot et al., 2006; Vamadevan et al., 2010).

The intestinal epithelial cells are supported by an array of immune and non-immune cells within the GI mucosa and within the gut-associated lymphoid tissues (GALT) that play important roles such as excluding luminal pathogens, suppressing inappropriate inflammation, responding against invading pathogens, and promoting wound healing to maintain the intestinal barrier functions (Cario et al., 2007; Coombes et al., 2007; Kim et al., 2018; Lycke et al., 1987; Silvey et al., 2001). Loss of this barrier function leads to increased non-specific translocation of GI luminal contents including the microbes, which could trigger PRRs on epithelial cells and mucosal immune cells to induce inflammation, leading to GI tract disorders such as IBD. Because of their nearly ubiquitous presence amongst many host organisms from *Drosophila* to humans, GI tract commensals are thought to have co-evolved with the host and are thought to harbor many mutually beneficial processes such as promoting gut barrier functions and maintaining immune homeostasis. This is highlighted by the observations that immune disorders such as IBD are associated with dysbiosis (Willing et al., 2009), although the mechanism behind the phenomena is not clear. While there are a lot of mechanisms by

which commensals probably do this, the majority of the studies on probiotics have focused on three major mechanisms the host utilizes to maintain the gut barrier that commensals are able to reinforce: strengthening the epithelial tight junctions, promoting mucus and antimicrobial secretions, and modulating IgA responses as described in **Table 1**.

Mechanism	Example probiotics
Direct competition	<i>Lactobacillus acidophilus</i> , <i>L. salivarius</i> , fecal transplantation
Strengthening gut epithelial barrier	<i>L. acidophilus</i> , <i>Streptococcus thermophilus</i> , <i>L. rhamnosus</i> GG, <i>Bifidobacterium infantis</i>
Induce host secretions	<i>Lactobacillus plantarum</i> strain 299v, <i>L. rhamnosus</i> GG, VSL#3, <i>Escherichia coli</i> Nissle 1917
Modulate host IgA	<i>Lactobacillus casei</i> , <i>L. rhamnosus</i> GG

**Table 1: Major Mechanisms by which Probiotics can Exclude Pathogens in the GI Tract.**

### Modulation of Epithelial Cell Barrier Functions

As previously described, the GI lumen is lined by a single layer of intestinal epithelial cells that are joined together by tight junctions, adherens junctions, and desmosomes. Desmosomes, or macula adherens, are cell-cell junction plaques that anchor intracellular intermediate filaments between cells, providing structural integrity for the epithelial monolayer. Adherens junctions, or zonula adherens, are actin-anchoring cell-cell junctions that utilize calcium-dependent cadherin-catenin interactions that not only join neighboring cells together but also promote apical and basolateral polarization of the intestinal epithelium. Lastly, tight junctions, or zonula

occludens, are the most apical of the cell-cell adhesions that facilitate selective transport of ions and solutes and also prevent translocation of microbes and toxins. Of the adhesive junctions, tight junctions are the best characterized and have been the subject of studies related to probiotics. Probiotics can enhance the functions of tight junctions, which would strengthen the epithelial barrier integrity. Overall functions of tight junctions can be estimated *in vitro* by measuring electrical resistance across the epithelial monolayer, or transepithelial resistance (TER). For example, probiotic strains of *L. acidophilus* and *Streptococcus thermophilus* slightly increased TER in Caco-2 cells, a human colonic epithelial cell line (Resta-Lenert and Barrett, 2003). In the same study, the probiotics helped HT-29 cells, another human colonic cell line, maintain TER levels during infection with enteroinvasive *E. coli* (EIEC), which normally results in a 3-fold loss in TER within 24 hours. This correlated with a reduction in the number of EIEC colony forming units (CFU) that passed through the epithelial cells, suggesting that the probiotics help keep the invasive pathogen at bay by strengthening the epithelial tight junctions (Resta-Lenert and Barrett, 2003). Tight junctions consist of four families of proteins: occludins, claudins, junctional adhesion molecules, and tricellulins (Furuse et al., 1993; 1998; Ikenouchi et al., 2005; Stevenson et al., 1986). Occludins are regulated through phosphorylation that helps localize these proteins to the tight junctions to link the junction to the actin cytoskeleton (Andreeva et al., 2001). Treatment of HT-29 cells with *L. acidophilus* and *S. thermophilus* resulted in an increase in occludin and zona occludens protein 1 (ZO-1) phosphorylation suggesting that the probiotics function through modulation of tight junction proteins (Resta-Lenert and Barrett, 2003). Live probiotics were required for these effects implying that there are some effector molecules produced by the probiotics, but the molecules remain unidentified. In

addition, these probiotics induced phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2) (Resta-Lenert and Barrett, 2003), but how the probiotics influence this pathway and what other pathways are influenced remain to be elucidated. It should be noted that there is no evidence yet for these probiotics influencing tight junctions *in vivo*. One concern for these probiotics is that spent medium from *L. acidophilus* and *S. thermophilus* cultures were unable to influence tight junctions *in vitro*, suggesting a requirement for close contact of live bacteria to the epithelial cells (Resta-Lenert and Barrett, 2003). In the GI tract, the majority of commensals are thought to be spatially separated from the epithelium with a layer of mucus secreted by the goblet cells (Johansson et al., 2008), and it is unclear if probiotics could come in close contact with the epithelial cells and affect tight junctions *in vivo*. A mucus-producing clone of HT-29 cells (HT-29-MTX) is available (Gagnon et al., 2013) and could be used to determine if a probiotic could affect tight junctions in the presence of host mucus.

Barrier dysfunctions have been well-documented in many inflammatory conditions of the mucosa (Johansson et al., 2014; Meddings et al., 1999), and inflammatory mediators such as interferon gamma (IFN- $\gamma$ ) and tumor necrosis factor alpha (TNF- $\alpha$ ) have been demonstrated to increase epithelial monolayer permeability and decrease TER in many epithelial cell lines (Fish et al., 1999; Madara and Stafford, 1989; Schmitz et al., 1999). *Bifidobacterium infantis* is a probiotic present in the VSL#3 formulation and *B. infantis*-conditioned medium prevented TER loss induced by IFN- $\gamma$  and TNF- $\alpha$  in T84 cells, another human colon cancer cell line (Ewaschuk et al., 2008). *B. infantis*-conditioned medium did this using a similar mechanism as *L. acidophilus*: increase in TER, increased levels of claudins and occludins, and phosphorylation of

ERK1 and 2. Ewaschuk and colleagues were able to assess the effects of *B. infantis* *in vivo* by measuring *ex vivo* tissue permeability of colons isolated from mice treated with *B. infantis*. They found that *B. infantis* reduced intestinal permeability and that these effects correlated with beneficial effects, as *B. infantis* reduced colitis due to interleukin-10 (IL-10)-deficiency (Ewaschuk et al., 2008). However, splenocytes from *B. infantis*-treated mice produced reduced levels of IFN- $\gamma$ , and colonic IFN- $\gamma$  levels were indeed reduced as well, making it difficult to know if the reduction in colitis by *B. infantis* administration was due to direct improvement on tight junction functions or due to a decrease in inflammatory cytokines. Modulation of immunity by probiotics is a subject of further discussion, but this study demonstrates that a probiotic can reinforce tight junction functions *in vivo* to benefit hosts during inflammation-induced colitis (Ewaschuk et al., 2008).

Some mechanisms by which probiotics reinforce the intestinal barrier functions do not necessarily involve the tight junctions. The intestinal epithelial cells are subject to high rates of turnover, constantly being shed from the luminal surface, and then replaced by proliferation and differentiation of the stem cells found in the crypts (Bjerknes and Cheng, 1981). Noxious stimuli such as inflammation can cause death of GI epithelial cells to disrupt the barrier and trigger an inflammatory reaction that further increases intestinal permeability. Specifically, TNF induces epithelial cell apoptosis, a programmed form of cell death, in cell lines and in mouse colon explants which can be prevented by conditioned medium from *L. rhamnosus* GG (Yan et al., 2007). *L. rhamnosus* GG does this by activating protein kinase B (Akt) that mediates cellular pro-survival signals, and this activity was attributed to two unidentified proteins of 75 and 40 kilodaltons (kDa) in size (Yan et al., 2007). There are many mechanisms by

which probiotics can limit epithelial permeability, and through these, probiotics can help exclude pathogens from invasion. Still, the specific molecules that mediate these effects remain mostly unidentified, and elucidating the mechanisms, especially the signaling pathways by which epithelial barriers are reinforced, would help identify targets for therapies in many inflammatory GI conditions.

### **Modulation of Epithelial Secretions**

It is essential to recognize that the epithelium is not just a physical barrier, but also an active participant in regulating the intestinal microbiota. To this end, the epithelial layer hosts specialized cell types that serve specific functions. Goblet cells, first noted by Friedrich Henle in 1837 and noted for mucus production by Franz von Leydig in 1857, are one such cell type, forming a gel-like layer that covers the luminal surface of the GI epithelium. In the colon, the mucus layer is divided into the inner and outer layers. The inner layer is anchored to the goblet cells and is generally thicker, with estimated pore sizes around 0.5  $\mu\text{M}$ , which prevent penetration by most bacteria (Johansson et al., 2008). About 50  $\mu\text{M}$  (mice) or 200  $\mu\text{M}$  (humans) away from the epithelium, the mucus is converted, through proteases, into a thinner outer layer that allows penetration by bacteria. This two-layer system is thought to prevent most bacteria from coming in direct contact with the epithelium, preventing intestinal inflammation. The mucus layers consist of about 50 proteins, and mucin 2 (Muc2) is the most critical, as Muc2-deficient mice lack colonic mucus (Johansson et al., 2014). Muc2 is heavily polymerized and glycosylated, eventually forming a massive web-like structure that gives mucus the sticky nature with pore sizes designed to limit bacterial penetration (Ambort et al., 2012). Muc3 is another mucin protein of the small intestine thought to be large and heavily glycosylated as well (Gum et al., 1997), though the exact function is not



known. Germ-free mice have inner mucus layers that are penetrated by microbe-sized beads (Johansson et al., 2015), demonstrating that commensals are essential for proper formation of the mucus layer. Probiotics can reinforce this mucus layer, which would help exclude pathogens from invading the intestinal mucosa. Multiple probiotic *Lactobacillus* species have been shown to increase expression of Muc3 in HT-29 cells, which correlated with reduced enteropathogenic *E. coli* (EPEC) adherence to the cells (Mack et al., 2003). VSL#3, a probiotic formulation of multiple organisms, including several *Lactobacilli* (Gionchetti et al., 2000), also increased expression of multiple mucin components by HT-29 cells (Otte and Podolsky, 2004). In this study, pharmacologic inhibition of mitogen-activated protein kinase (MAPK) prevented this increase, indicating that the probiotic mixture affects mucin production through specific signals. However, it is not clear how VSL#3 activates the MAPK pathway, and it is not clear if any other pathways are involved. It should be noted that no probiotics to date have been assessed for their ability to influence goblet cell functions. While intestinal cell lines are notorious for being heterogeneous and mucus-producing clones of these cell lines have been identified (Gagnon et al., 2013), the major mucus-producing cell type is the goblet cell, and this warrants further study. In addition, the ability of specific probiotics to influence the mucus layer *in vivo* has not been assessed. Goblet cells are known to be heavily regulated by the immune system (Oeser et al., 2014; Turner et al., 2013), and given the influence of commensals on immune homeostasis, it would not be surprising if probiotics can modulate goblet cell functions through immune modulation *in vivo*.

Mucus is not the only secretion at the GI epithelial barrier. Antimicrobial peptides (AMP) are oligopeptides with antimicrobial activity, produced by members

from all kingdoms of life. Since their discovery (Dubos, 1939), more than 5,000 AMPs have been discovered or synthesized (Zhao et al., 2013), and a variety of mechanisms exist by which they exhibit antimicrobial activity. In the human colon,  $\beta$ -defensins are the best characterized AMPs. The human genome encodes 33  $\beta$ -defensins, of which  $\beta$ -defensin 1, 2, and 3 are the most extensively studied.  $\beta$ -defensins are small cationic, cysteine-rich peptides of about 4 to 5 kDa in size with multiple disulfide bridges between the cysteine pairs (Klüver et al., 2005; Schibli et al., 2002). Because of their cationic nature,  $\beta$ -defensins preferentially bind to microbial membranes that are negatively charged; mammalian cell membranes tend to be more electroneutral. Once bound to the microbial surface,  $\beta$ -defensins penetrate through the lipid bilayer, forming a pore through which intracellular contents leak, resulting in cell death (Lehrer et al., 1989). Probiotics could act by inducing host secretion of AMPs to reinforce antimicrobial defenses at the gut barrier. *E. coli* Nissle 1917 and other probiotic strains of *E. coli* have been demonstrated to increase fecal levels of  $\beta$ -defensin 2 in humans after 3 weeks of probiotic administration, and also induced expression of  $\beta$ -defensin 2 messenger RNA (mRNA) in Caco-2 cells *in vitro* (Möndel et al., 2008). While this demonstrates that probiotics have the potential to reinforce host antimicrobial defenses through AMPs, it is not clear if AMP secretion is responsible for the exclusion of pathogens from the GI tract *in vivo*. In addition, probiotic strains of *E. coli* are also susceptible to  $\beta$ -defensins, and the induction of  $\beta$ -defensin in humans by probiotics is transient, as  $\beta$ -defensin levels in feces start to return to baseline by about 9 weeks after treatment (Möndel et al., 2008). Still, probiotics have the ability to promote host secretions, including mucus and AMPs, and through these mechanisms, they can help exclude pathogens from invading the mucosal surfaces.

## **Modulation of Secretory IgA**

The third major mechanism by which probiotics can promote gut barrier functions involves a major component of the mucosal immune system, secretory IgA (sIgA). In the serum, IgA is primarily in its monomeric form. In the mucosa, IgA is primarily in its dimeric form: two monomers joined together by the J chain (Halpern and Koshland, 1970; 1973). IgA is the most abundant antibody class, representing about 70% of antibodies made by mammals (Chodirker and Tomasi, 1963). To this end, the GI tract is lined ubiquitously with GALT such as Peyer's patches in the ileum important for the development of IgA-producing cells (Craig and Cebra, 1971). The IgA-secreting plasma cells then populate the GI lamina propria, where they are thought to produce IgA. The dimeric IgA is bound by the polymeric Ig receptor (pIgR) expressed on epithelial cells that mediates basolateral to apical transcytosis of dimeric IgA (Brandtzaeg, 1974; Brandtzaeg and Prydz, 1984; Tomasi et al., 1965; Tourville et al., 1969). Close to the luminal surface, IgA is then released from the pIgR and secreted from the epithelium as sIgA, associated to a part of the pIgR known as secretory component (Brandtzaeg, 1974; Tourville et al., 1969). Canonically, it is believed that sIgA serves as the first-line defense against mucosal pathogens, neutralizing microbial toxins and contributing to pathogen exclusion by binding to microbes and limiting their penetration through the mucus layer (Boullier et al., 2009; Lycke et al., 1987; Silvey et al., 2001). Commensals are important for the production of IgA since germ-free mice had no detectable serum IgA and slightly reduced cecal sIgA levels that was restored to levels found in conventional mice once commensals were introduced (Benveniste et al., 1971). Germ-free mice also had reduced numbers of IgA plasma cells in the duodenum that were also restored to conventional levels by introducing certain microbes (Moreau

et al., 1978), indicating that the microbes stimulate sIgA production by promoting the development of IgA-producing cells. Certain probiotics such as *L. casei* have been demonstrated to increase IgA levels in the GI tract by increasing the number of IgA<sup>+</sup> cells in the small intestines after about a week (Galdeano and Perdígón, 2006). However, this IgA was not specific to the administered probiotic, suggesting that *L. casei* generally increases IgA levels rather than inducing an antigen-specific antibody response (Galdeano and Perdígón, 2006). By reinforcing sIgA production in the GI tract, probiotics have the potential to protect hosts from invading pathogens.

While it is important to note that sIgA mediates important canonical functions within the GI tract such as neutralization of microbial toxins and exclusion of pathogenic microbes (Boullier et al., 2009; Lycke et al., 1987; Silvey et al., 2001), how a non-specific increase in sIgA levels would benefit hosts is not immediately clear. In addition, it is not clear what mechanisms actually drive increased sIgA levels in response to commensals or probiotics. A potential explanation for how probiotics could protect hosts through IgA stems from a recent appreciation for how commensals influence the IgA repertoire. The diversity of antibody specificities is generated in large part by the action of activation-induced cytidine deaminase (AID) in activated B cells (Muramatsu et al., 2000). AID functions by converting deoxycytidines to deoxyuracil in DNA of the Ig variable (V) genes, which results in the introduction of a transition mutation from a cytidine-guanine pair to a thymine-adenine pair, or error-prone excision repair of the deoxyuracil, that introduces mutations, diversifying the V gene pool as B cells proliferate (Petersen-Mahrt et al., 2002); this process is called somatic hypermutation (SHM). AID is also responsible for class switch recombination, which is required to generate different antibody classes, including IgA (Muramatsu et al., 2000).

Commensal microbes increase the levels of sIgA in the gut (Benveniste et al., 1971), but this is only one aspect of what commensals do to influence sIgA production. In germ-free mice, the level of sIgA is decreased, but also decreased is the frequency of SHM, indicating that commensals also influence the generation of sIgA diversity (Lindner et al., 2012). The ability to diversify the sIgA repertoire is likely more important than increasing sIgA levels to maintain gut homeostasis, since AID mutant mice that have defective AID functions had levels of fecal sIgA comparable to that of wild-type (WT) mice but had reduced sIgA diversity that correlated with expanded intestinal bacterial population and increased invasion by *Yersinia enterocolitica* during infection (Wei et al., 2011). The reduction in SHM frequency is also observed in T cell-deficient mice in conventional housing, indicating that commensals drive sIgA diversification through T cells (Lindner et al., 2012). Generally, microbes are sampled through specialized epithelial cells called microfold (M) cells that bring in luminal microbes through transcytosis to the associated immune cluster (Bockman and Cooper, 1973; Owen and Jones, 1974). Here, antigen presenting cells such as dendritic cells (DC) take up the microbe and activate T cells that coordinate germinal center reactions to give rise to antibody responses (Alpan et al., 2001).

Activated T cells can differentiate into T follicular helper ( $T_{FH}$ ) cells that are critical for selective survival of high-affinity B cells and the maintenance of germinal centers (Breitfeld et al., 2000). The germinal center also is home to T follicular regulatory ( $T_{FR}$ ) cells that regulate the germinal center reactions (Chung et al., 2011). These follicular T cells help shape the sIgA repertoire against intestinal microbes, as evidenced by the change in the profile of IgA-coated intestinal microbes by the transfer of follicular T cells into immunodeficient mice (Kawamoto et al., 2014). Therefore,

probiotics could promote the development of antigen-specific sIgA responses that could target pathogens for exclusion. An example of this is observed with *B. bifidum* and *B. infantis* that reduce rotavirus-induced diarrhea in mice which correlates with increased levels of virus-specific mucosal sIgA levels (Qiao et al., 2002).

The specific mechanisms for how the administration of probiotics leads to the generation of antibody responses against seemingly unrelated pathogens are not clear, but two possibilities seem feasible. For one, the probiotics could harbor some antigens that bear a resemblance to those found on pathogens, therefore driving a probiotic-specific sIgA response that also targets pathogens with similar epitopes, a phenomenon that could be described as molecular mimicry. However, probiotics do not usually induce a sIgA response targeting itself, as the administration of *L. casei* increased sIgA responses but none that targeted *L. casei* itself (Galdeano and Perdígón, 2006). Another possibility is that, by stimulating GALT immune cells including DCs and follicular T cells, overall mucosal antibody responses may be heightened, increasing the likelihood of generating antigen-specific antibody responses against others, perhaps pathogens like rotavirus. Unfortunately, the increase in sIgA by probiotics is a generally accepted (and marketed) benefit that needs further mechanistic understanding, and how it would actually be beneficial remains to be studied.

A recent observation about sIgA is that a part of the sIgA repertoire is innate-like, enriched for polyreactive antibodies, and thought to serve as a first-line defense mechanism (Bunker et al., 2017). The natural sIgA arises through a T cell-independent process and is not affected by the absence of germinal center reactions or SHM (Bunker et al., 2015). The natural IgA coats specific members of the commensal microbiota (Bunker et al., 2015; 2017), though what functional outcome this has on host physiology

is not clear. However, sIgA can enter the Peyer's patches in both *in vitro* and *in vivo* models, where they can be internalized by DCs (Rey et al., 2004), though functional outcomes on DCs or germinal center reactions were not assessed in this study. This suggests that sIgA-coated commensal microbes could selectively be transported to the GALT where they could interact with immune cells to affect host physiology. IgA-coated probiotic *L. rhamnosus* can stimulate DCs isolated from Peyer's patches to induce regulatory T cells (T<sub>reg</sub>) through IL-10 and transforming growth factor beta (TGF- $\beta$ ) (Mikulic et al., 2016). While the ability of IgA-coated *L. rhamnosus* to enter Peyer's patches *in vivo* or *ex vivo* was not assessed, this study suggests that sIgA, given that a part of its repertoire selectively binds to commensals, could mediate uptake of probiotics into the GALT where it can influence immune homeostasis. A similar phenomenon has been described for invasion-deficient *Salmonella* as well. While invasion-deficient *Salmonella* is avirulent, it is generally not considered a probiotic. Still, *in vivo* administration of IgA-coated invasion-deficient *Salmonella* by direct injection into the duodenum of mice resulted in increased presence of bacteria within the Peyer's patches compared to injection of uncoated bacteria, and this led to the generation of an antigen-specific IgA response (Fransen et al., 2015), demonstrating that IgA can mediate selective uptake of GI bacteria to promote antigen-specific IgA responses. Further investigation into the relationship between sIgA, commensals, and the overall IgA repertoire will be needed to understand the basis by which probiotics can protect hosts from pathogens through sIgA.

## **Probiotic Mechanism of Action: Immune Homeostasis**

### **Microbes and Immune Homeostasis**

While a substantial number of studies have focused on ways probiotics confer protection against pathogens through exclusion, it is essential to note that this is not the only way probiotics can benefit hosts. In fact, probiotics are currently marketed with claimed health benefits that span the entire human body: improved digestion, strengthened immune system, reduced allergy, improved cardiovascular functions, and even improved psychological health. One particular benefit that is becoming a major focus of multiple research disciplines is the effect of the microbiota on immune homeostasis. Mucosal surfaces, especially the GI tract, are in constant contact with microbes that need to be regulated by host immunity. Failure to regulate the intestinal microbes can leave the host vulnerable to pathogenic invasion (Wei et al., 2011). However, inappropriate activation of immunity has detrimental effects for the host, as evidenced by inflammatory diseases caused by dysregulation of host immunity such as IBD (Hugot et al., 2001; Kühn et al., 1993). Therefore, a delicate balance between the need to restrict microbes and to limit inflammation must be achieved. To this end, many host mechanisms exist to regulate immune responses tightly. We have already discussed the influence of the microbiota on host physiology, but the relationship between host and commensal microbes is suspected to be more intertwined. One evidence for this is the observation that the development of the different arms of the immune system, especially adaptive immunity, coincides with the evolutionary appearance of organisms that host a complex microbiota (Pancer et al., 2004; Zuo et al., 2017). Therefore, it is perhaps not surprising to see that the microbiota exerts its influence on just about every major host immunoregulatory mechanisms. Probiotics also target these



immunoregulatory mechanisms, mainly those described in **Table 2**. By reinforcing the immunoregulatory mechanisms, probiotics have the ability to promote immune homeostasis and prevent inflammatory diseases.

Mechanism	Role in homeostasis	Microbial influence
Regulatory T cells	Ag-specific tolerance, limit T effector functions, IL-10 production	Microbes (ex. PSA) increase IL-10 <sup>+</sup> T <sub>reg</sub> , SCFA induces IL-10 <sup>+</sup> T <sub>reg</sub>
MΦs	Wound-healing, limit T cell responses, IL-10 production	<i>H. hepaticus</i> polysaccharide induce anti-inflammatory signature, SCFA induces anti-inflammatory MΦ
iNKT Cells	Limit infection, promote wound healing phenotype in recruited MΦ	<i>B. fragilis</i> sphingolipid reduces iNKT-mediated inflammation

**Table 2: Major Mechanisms by which Probiotics Promote Immune Homeostasis**

### Modulation of Regulatory T Cells

The major immune cell type that mediates regulation of immunity is the T<sub>reg</sub> cell, generally defined by the expression of the master transcription factor, forkhead box protein P3 (Foxp3) (Fontenot et al., 2003; Hori et al., 2003). Foxp3 expression by T<sub>reg</sub> cells is critical to maintain tolerance to self-antigens, as Foxp3-deficient mice experience systemic autoimmune disease and die by about 4 weeks of age (Fontenot et al., 2003). In humans, mutations in *FOXP3* gene are linked with the systemic autoimmune disease known as immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) (Bennett et al., 2001). There are two main subtypes of T<sub>reg</sub> cells, natural T<sub>reg</sub> (nT<sub>reg</sub>) cells and inducible T<sub>reg</sub> (iT<sub>reg</sub>) cells. nT<sub>reg</sub> cells develop from the thymus through the host-intrinsic mechanism and generally mediate central tolerance against self-antigens upon release into the periphery. Certain peripheral naïve T cells can be induced into iT<sub>reg</sub> cells, which then can mediate tolerance against both self- and foreign-antigens.

Unlike nT<sub>reg</sub> cells, iT<sub>reg</sub> cells are heavily influenced by the local cytokine milieu, especially TGF- $\beta$ , which is thought to help maintain Foxp3 expression in T<sub>reg</sub> cells (Zheng et al., 2010). The canonical anti-inflammatory cytokine IL-10 is also known to potentiate iT<sub>reg</sub> differentiation (Hsu et al., 2015). The GI tract is perhaps the most critical place to maintain immune homeostasis, and the balance of effector T cells and T<sub>reg</sub> cells is critical. This was experimentally demonstrated by the transfer of naïve T cells, defined by the expression of cluster of differentiation (CD) markers as CD4<sup>+</sup>CD45RB<sup>high</sup>, to immunodeficient mice which recreated a T<sub>reg</sub> cell-deficient host and resulted in pan-GI tract inflammation (Read et al., 2000), assumed to be due to unchecked T cell activation. In this model, co-transfer of the CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>low</sup> compartment, known to be enriched for T<sub>reg</sub> cells, ameliorated inflammation in the GI tract (Read et al., 2000), suggesting that T<sub>reg</sub> cells are important to maintain homeostasis in the GI tract. In the colon, the majority of the T<sub>reg</sub> cells are iT<sub>reg</sub> cells (Atarashi et al., 2011; Geuking et al., 2011; Lathrop et al., 2011), and since they are influenced by environmental signals, they are thought to be the cells interacting with the microbiota to maintain immune tolerance. The microbiota is known to play a role in promoting or maintaining T<sub>reg</sub> cell population within the GI tract since germ-free mice harbor reduced numbers of T<sub>reg</sub> cells in the colonic lamina propria (Geuking et al., 2011). Not surprisingly, a significant way probiotics are thought to function in homeostasis is to reinforce the T<sub>reg</sub> cell compartment. Chronic administration of *Bifidobacterium breve* Yakult strain to mice ameliorated colitis in the naive T cell transfer model, and this is thought to be due to an increase in the number of colonic T<sub>reg</sub> cells that produce IL-10 (Jeon et al., 2012). It should be noted here that *B. breve*-induced IL-10<sup>+</sup> T<sub>reg</sub> cells did not express Foxp3, a

phenotype consistent with a subset of T<sub>reg</sub> cells called type 1 T<sub>reg</sub> cell (T<sub>1</sub>) (Groux et al., 1997).

The effect of microbes on immune homeostasis and T<sub>reg</sub> cells is often studied in the context of the GI tract, but the effects are not necessarily confined locally. Certain *Clostridium* species of the GI tract microbiota are known to be sufficient to induce IL-10-producing, Foxp3<sup>+</sup> T<sub>reg</sub> cells in the colon in germ-free mice (Atarashi et al., 2011). Supplementation of these *Clostridia* species to normal mice under specific pathogen-free (SPF) conditions limited colitis induced by the chemical irritant, dextran sodium sulfate (DSS) (Atarashi et al., 2011), indicating that the local induction of T<sub>reg</sub> cells by the microbe helps limit inflammation. However, mice supplemented with these *Clostridia* species were also resistant to allergic sensitization as well, as these mice had reduced serum levels of ovalbumin- (OVA) specific IgE following systemic immunization with OVA (Atarashi et al., 2011).

The mechanisms by which microbes in the GI tract can have systemic immunomodulatory effects are not clear, but two main possibilities exist. For one, locally induced T<sub>reg</sub> cells, or any other immune cell, could mediate systemic effects either by the production of systemic immunoregulatory mediators or by redistribution to other tissues. The other possibility is that the mediator molecules of microbes drain into the circulation and directly affect immune cells in other tissues. In *Clostridia*-supplemented mice, splenocytes produced higher levels of IL-10 upon stimulation, which suggests that IL-10<sup>+</sup> T<sub>reg</sub> cells are present systemically (Atarashi et al., 2011). Still, it is not clear if this increased IL-10 production even comes from T<sub>reg</sub> cells, nor is it clear if these cells originated from the GI tract. However, there is compelling evidence for microbial products directly affecting tissues outside the GI tract.

Short chain fatty acids (SCFA) are byproducts of microbial fermentation of carbon sources, often those that cannot be processed by the mammalian hosts such as dietary fiber. Administration of SCFAs to germ-free mice resulted in an increase in intestinal T<sub>reg</sub> cells and protected mice from colitis in the T cell transfer colitis model (Smith et al., 2013), indicating that microbes promote immune homeostasis by inducing T<sub>reg</sub> cell differentiation through SCFAs. SCFAs are known to directly promote T<sub>reg</sub> cell differentiation *in vitro* through G-protein coupled-receptors (GPCR) (Smith et al., 2013), and SCFAs can be detected in the human circulation (Cummings et al., 1987), suggesting that, by getting into the circulation, SCFAs could directly engage T cells for T<sub>reg</sub> cell differentiation in distant tissues. Indeed, one study demonstrated that administration of *L. rhamnosus* GG to mice resulted in an expansion of SCFA-producing *Clostridia*, which increased T<sub>reg</sub> cells in the bone marrow to promote bone homeostasis (Tyagi et al., 2018). Therefore, by enhancing the T<sub>reg</sub> compartment, probiotics can help maintain immune homeostasis within the local environment and beyond.

While probiotics promote T<sub>reg</sub> cell differentiation, they do not necessarily target T cells directly. In fact, the best-characterized probiotics have been found to exert their functions through a mediator cell type, generally of the myeloid lineage, which can promote T<sub>reg</sub> cell differentiation. The classic example is an anaerobic member of the microbiota, *Bacteroides fragilis*. *B. fragilis* is known to ameliorate colitis in multiple murine models, and as in many other probiotic agents, this is mediated through the induction of IL-10-producing T<sub>reg</sub> cells (Mazmanian et al., 2008). This protective effect requires polysaccharide A (PSA), a zwitterionic polysaccharide that forms the bacterial capsule that recapitulates protection from colitis when administered as purified PSA (Baumann et al., 1992; Mazmanian et al., 2008). PSA inhibits the production of TNF- $\alpha$

induced by infection with *Helicobacter hepaticus* in *in vitro* co-cultures of bone marrow-derived DCs (BMDC) and T cells in a Toll-like receptor 2 (TLR2)-dependent manner (Mazmanian et al., 2008). *In vivo* protection from colitis also requires TLR2 (Mazmanian et al., 2005; Round and Mazmanian, 2010; Wang et al., 2006), indicating that PSA exerts its effects in part through TLR2 on DCs. Other probiotics also go through mediator cells to induce T<sub>reg</sub> cells. *B. breve* Yakult strain induces T<sub>ri</sub> cells in the colon and *in vitro* during co-culture of intestinal CD103<sup>+</sup> DCs and splenic CD4<sup>+</sup> T cells, a process that requires TLR2 signaling in the DCs (Jeon et al., 2012). Non-immune cells can also be the mediator cell type for promoting T<sub>reg</sub> cell differentiation. In the case of SCFA-producing microbes, SCFAs can act on pancreatic  $\beta$  cells, the insulin-producing cell, to induce production of cathelicidin-related AMP (CRAMP) which promotes anti-inflammatory polarization of macrophages (M $\Phi$ ), leading to increased T<sub>reg</sub> cell differentiation that correlates with protection from autoimmune diabetes (Sun et al., 2015).

### **Modulation of Macrophages**

Other immune cells can also be targeted by microbes to promote immune homeostasis. In particular, M $\Phi$ s are a diverse group of cells of the mononuclear phagocyte (MNP) system and tissue resident cells that perform homeostatic functions in virtually all tissues (Broug-Holub et al., 1997; Fox et al., 1987; Gautier et al., 2012; Geissmann et al., 2003; Mackaness, 1962; Nahrendorf et al., 2007; Nimmerjahn et al., 2005; Udagawa et al., 1990). Classically, M $\Phi$ s can be characterized in two major categories, classically activated M1 M $\Phi$ s that mediate pro-inflammatory type I responses and alternatively activated M2 M $\Phi$ s that mediate pro-fibrotic type II responses (Mills et al., 2000). Under *in vitro* conditions, M $\Phi$ s can be polarized into M1 and M2 M $\Phi$ s in

response to the type I cytokine IFN- $\gamma$  and a microbial ligand or type II cytokines IL-4 and IL-13, respectively (Doherty et al., 1993; Held et al., 1999; Murray et al., 1985; 2014; Stein et al., 1992). M1 M $\Phi$ s are best known for their antimicrobial functions and their expression of inducible nitric oxide synthase (iNOS). M1 M $\Phi$ s phagocytose microbes and kill them by producing toxic molecules like reactive nitrogen species (RNS) through iNOS and reactive oxygen species (ROS) through the action of nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase, a process characterized as the respiratory burst (Sbarra and Karnovsky, 1959; Segal, 1987; Xie et al., 1992). M2 M $\Phi$ s are typically associated with immunoregulation and anti-parasite responses, typically defined by the expression of arginase 1 (Arg-1) (Munder et al., 1998). M2 M $\Phi$ s have been studied often in tumor environments, specifically designated tumor-associated M $\Phi$ s since these cells are now recognized as a distinct subtype, with robust suppressive activity against tumor-specific T cells (Curiel et al., 2004; Kuang et al., 2009; Torroella-Kouri et al., 2009). M2 M $\Phi$ s are thought to suppress T cell functions by three different mechanisms: inhibitory cytokines, inhibitory cell surface ligands, and local depletion of arginine (Curiel et al., 2004; Huber et al., 2010; Modolell et al., 2009; Pesce et al., 2009; Taylor et al., 2006). Both iNOS and Arg-1 use arginine as the substrate, and this is thought to drive polarization of M $\Phi$ s since these enzymes mutually exclude the action of the other by competing for the same substrate (Munder et al., 1998). However, it is not known how these enzymes contribute to differential polarization of M $\Phi$ s, and there are other processes known to influence this polarization such as  $\beta$ -oxidation of fatty acids, a process important for M2 polarization (Huang et al., 2014). The designation of M $\Phi$ s into M1 and M2 originally fit well with the two classes of T cell responses (Mills et al., 2000), but this likely represents an oversimplified view of M $\Phi$ s. In fact, other signals that

activate MΦs such as IL-10, TGF-β, and glucocorticoids did not fit with the original descriptions of M1 and M2 MΦs, and since these signals induced mostly immunoregulatory functions like that of M2 MΦs, the M2 MΦs were proposed to be further subdivided into subsets based on activating signals (Anderson and Mosser, 2002; Edwards et al., 2006; Ehrchen et al., 2007). Still, the factors that affect MΦ phenotypes are a seemingly endless list, including salt concentrations (Binger et al., 2015) and even cell shape (McWhorter et al., 2013), and attempts to subdivide MΦs into more subsets would be equally complex. This is further complicated *in vivo* due to the different developmental ontogeny of MNPs, including MΦs *in vivo*. The circulating MNP progenitors, monocytes, arise from the bone marrow and differentiate into MΦs and DCs upon being recruited to tissues (Tamoutounour et al., 2013; Volkman and Gowans, 1965). However, different tissues harbor resident MΦs that are thought to arise from progenitors during fetal and yolk sac lymphopoiesis (Ghosn et al., 2010; Ginhoux et al., 2010; Hoeffel et al., 2012; De Schepper et al., 2018; Tamoutounour et al., 2013). Given the recent appreciation of the complex nature of MNPs *in vivo*, the most appropriate view is likely that MΦs exist in a dynamic spectrum of specialized cells with differing ontogeny that constantly modulate their functions in response to environmental cues.

The GI tract harbors many MΦs, typically expressing C-X<sub>3</sub>-C chemokine receptor 1 (CX<sub>3</sub>CR1) (Geissmann et al., 2003). The mucosal layer receives constant input from the circulating monocytes that replenish the MΦ pool while the deeper muscle layers have tissue resident MΦs that self-renew (De Schepper et al., 2018). Based on localization, it is thought that the mucosal MΦs mediate interactions with the microbiota while MΦs in the muscularis layer interact with the enteric neurons to support digestive functions of the GI tract (Gabanyi et al., 2016; De Schepper et al.,

2018). In fact, ablation of GI tract MΦs resulted in increased GI tract transit time, indicative of decreased GI motility (De Schepper et al., 2018). Intestinal MΦs play an important role in coordinating immune responses against microbes in the GI tract, as depletion of CX<sub>3</sub>CR1<sup>+</sup> MNPs resulted in increased T cell responses against intestinal pathogens (Kim et al., 2018), indicating that these cells limit inflammation under homeostatic conditions. The microbiota is known to influence these MΦs, since depletion of the same CX<sub>3</sub>CR1<sup>+</sup> MNPs resulted in decreased T cell responses against intestinal pathogens in antibiotic-treated mice (Kim et al., 2018), suggesting that under certain conditions, intestinal MΦs promote antimicrobial responses instead. Specific microbes can exploit MΦs to promote homeostasis as well. We already discussed SCFAs that induce pancreatic CRAMP to modulate MΦs and promote T<sub>reg</sub> cell differentiation, preventing autoimmune diabetes. CRAMP-induced pancreatic MΦs take on an immunoregulatory phenotype with high TGF-β production and expression of aldehyde dehydrogenase (ALDH), an enzyme involved in retinoic acid (RA) production, which is linked with T<sub>reg</sub> cell differentiation (Sun et al., 2015). Still, it should be noted that this study did not assess if CRAMP-induced regulatory MΦs were responsible for T<sub>reg</sub> cell differentiation, and the mechanism by which CRAMP confers this MΦ phenotype is not known. Another example is *H. hepaticus*, which was demonstrated to produce large polysaccharides that modulate MΦs to express an anti-inflammatory gene expression profile through TLR2, which prevented exacerbation of colitis (Danne et al., 2017). This study assessed signaling pathways needed for *H. hepaticus* polysaccharide *in vitro*, but these pathways were not confirmed *in vivo*. Nonetheless, these studies demonstrate that microbes target MΦs to reinforce their homeostatic functions and prevent inflammatory disease.



## **Modulation of Invariant Natural Killer T Cells**

Another cell type of interest is invariant natural killer T (iNKT) cells, tissue resident lymphocytes that recognize lipid antigens presented by the major histocompatibility complex (MHC) class I-like molecule, CD1d (Bendelac et al., 1995; Kawano et al., 1997). iNKT cells are distributed across many tissues, but they have been best characterized in mucosal tissues and adipose tissues. Like the T helper cell compartment, iNKT cells develop from the thymus and differentiate into IFN- $\gamma$ -producing iNKT1, IL-4-producing iNKT2, IL-17-producing iNKT17, follicular helper iNKT, and IL-10-producing regulatory iNKT10 compartments to coordinate different immune responses (Georgiev et al., 2016). In the liver, the site where iNKT cells are the most frequent, iNKT cells continuously patrol the liver sinusoids (Geissmann et al., 2005). During infection, the patrolling iNKT cells get activated and become stationary at the infective focus, producing pro-inflammatory mediators like IFN- $\gamma$  (Velázquez et al., 2008). iNKT cells are thought to then coordinate antimicrobial responses by other cells like Kupffer cells, the liver resident M $\Phi$ s, and help clear pathogens (Lee et al., 2010). However, one study demonstrated that during sterile focal liver injury induced by heat, iNKT cells get to the injury site but make a 180° turn from the injured focus and settle at the edge of injury site (Liew et al., 2017). There, iNKT cells produce IL-4 and not IFN- $\gamma$ , supposedly in response to self-lipid antigens. This was proposed to coordinate the differentiation of recruited monocytes to wound healing M $\Phi$ s through IL-4, as IL-4 blockade prevented wound healing (Liew et al., 2017). This suggests that under certain circumstances, iNKT cells promote wound healing in response to lipid antigens.

In the GI tract, iNKT cells represent about 1% of the lymphocyte population (Matsuda et al., 2000). Microbes have been suggested to influence the iNKT population

within the GI tract, as germ-free mice had increased numbers of colonic iNKT cells compared to mice in SPF conditions (Olszak et al., 2012). Specific microbes can have similar effects, as the administration of *B. fragilis* to germ-free mice was sufficient to reduce colonic iNKT cell numbers to levels found in mice under SPF conditions (An et al., 2014). *B. fragilis* does this through a specific sphingolipid molecule, GSL-Bf717, which limits iNKT cell activation *in vitro* and is required for reduced iNKT cell numbers *in vivo* (An et al., 2014). iNKT cells are known to drive allergic inflammation in the gut through the production of IL-13 in a chemical-induced allergy model (Heller et al., 2002), and SPF mice and *B. fragilis* monocolonized mice had reduced inflammation during oxazolone-induced colitis compared to germ-free mice (An et al., 2014; Olszak et al., 2012), suggesting that microbes reduce inflammation by modulating the gut iNKT cells. On the other hand, iNKT cells from germ-free mice are hyporesponsive, and exposure to *Sphingomonas yanoikuyae* was sufficient to restore iNKT maturity (Wingender et al., 2012), suggesting that certain microbes instead promote proper maturation of iNKT cells to reinforce host defenses. It should be noted that for common probiotic organisms, their effects on iNKT cells have not been explored. Since iNKT cells play roles in both host defenses and immunoregulation in response to microbes, they represent prime targets for probiotic therapy that remains to be explored.

### ***Bacillus subtilis*, the Probiotic**

#### **Exposure to *Bacillus subtilis***

*B. subtilis* is a gram positive, spore-forming bacterium ubiquitously found in the environment, in particular in the soil (Siala et al., 1974). Human exposure to *B. subtilis* is thought to occur through contact with soil and as a part of the diet in many cultures. Fermented soybean food products contain *B. subtilis*, the most widely recognized being

natto, a Japanese dish prepared by fermentation of cooked soybeans with a pure culture of *B. subtilis var. natto* (Ashiuchi et al., 1998). As a spore-forming bacterium that has been extensively studied by the scientific community, *B. subtilis* possesses qualities that give significant advantages as a potential probiotic product. Bacterial endospores are a dormant form of the cell that are generally formed under nutrient-depleted conditions. Once formed, spores can persist indefinitely, and can withstand a variety of extreme conditions including heat in excess of 100°C and even ultraviolet radiation (Alderton and Snell, 1969; Nicholson et al., 2005), which makes long-term storage of *B. subtilis* as a product fairly simple. Upon oral administration of spores, *B. subtilis* is known to complete its life cycle within the GI tract, from germination into a vegetative state to endospore formation prior to passage from the gut (Hoa et al., 2001). To date, oral administration of *B. subtilis* has not been associated with any adverse outcomes in immunocompetent individuals (Hoa et al., 2001), and *B. subtilis* has been safely consumed as part of fermented soybean products for a long time in certain regions of the world, making *B. subtilis* a durable probiotic easily administered orally, and safe for human consumption.

As an orally administered probiotic, *B. subtilis*-containing products have been tested for their potentially beneficial effects, though the specific mechanisms have not been elucidated. Like many other probiotics, some have suspected that *B. subtilis* can alter colonization of pathogens through competitive exclusion. One study tested if oral administration of *B. subtilis* PY79 spores could alter colonization by the murine enteric pathogen, *Citrobacter rodentium*, and found that *B. subtilis*-colonized mice had reduced the number of *C. rodentium* CFU by 75% and reduced diarrheal disease (D'Arienzo et al., 2006). A more recent study observed in a study in rural Southeast Asia

that people colonized with *B. subtilis* were not colonized with the opportunistic pathogen, *S. aureus* (Piewngam et al., 2018). This study then tested if *B. subtilis* prevents colonization by *S. aureus* by orally administering spores of *B. subtilis* NCIB 3610 to mice and found that this prevented intestinal colonization by *S. aureus*. This exclusion activity was attributed to *B. subtilis* production of fengycin, a cyclic lipopeptide, which interfered with *S. aureus* accessory gene regulator (Agr) quorum sensing critical for expression of various virulence factors (Ji et al., 1995; Piewngam et al., 2018).

### **Immune Modulation by *Bacillus subtilis***

Another way *B. subtilis* is thought to benefit hosts is through immune modulation. One study treated RAW264.7 MΦs with *B. subtilis* PY79 spores and found that the spores were taken up by the MΦs, which had slightly increased IL-6 gene expression (Duc et al., 2004). The authors also administered spores to mice orally and found an increase in gene expression of IFN- $\gamma$  and TNF- $\alpha$  in intestinal tissues and in GALT, suggesting that *B. subtilis* spores stimulate the immune system. In another study, *B. subtilis* A109 spores orally administered to mice were shown to result in increased MΦ oxidative burst activity when stimulated, and increased natural killer (NK) cell killing activity (Kosaka et al., 1998), indicating that *B. subtilis* stimulates multiple immune cells *in vivo*. Rhee and colleagues investigated the role of microbes in GALT development in rabbits and found that specific members of the microbiota, including *B. subtilis*, promote GALT development that is important for antibody repertoire diversification (Rhee et al., 2004). For the development of rabbit GALT, factors associated with stress responses in *B. subtilis* were required, though the mechanism behind this was not clear. In fact, how *B. subtilis* stimulates immune cells in all of the

studies discussed above is not known. Understanding how *B. subtilis* modulates the immune system, by identifying bacterial immunomodulatory factors and the corresponding host response pathways, will be critical for developing *B. subtilis* into a therapy that could be used in many clinical settings.

### ***Bacillus subtilis* Exopolysaccharide**

Our group was interested in understanding the mechanism by which *B. subtilis* benefits hosts, especially in the context of disease processes. The group initially chose the *C. rodentium*-induced murine colitis model where *B. subtilis* had been demonstrated to be protective (D'Arienzo et al., 2006), and confirmed the beneficial effect of *B. subtilis* in *C. rodentium* infection using an undomesticated, biofilm-forming strain of *B. subtilis*, NCIB 3610 (Jones and Knight, 2012). Bacterial biofilms are a community of bacterial cells encased within a self-produced extracellular matrix composed of carbohydrates, proteins, and nucleic acids. *B. subtilis* biofilms are also composed of a complex matrix, and the two major components are the protein TasA and the polysaccharide EPS produced by the products of the 15 gene *eps* operon, encoding *epsA* through *epsO* (Branda et al., 2001; 2006). Administration of a *B. subtilis epsH* mutant that fails to produce EPS could not protect mice from *C. rodentium*-induced colitis, suggesting that EPS is required for protection (Jones and Knight, 2012). Biofilm-associated bacteria are thought to be more persistent (Anwar et al., 1992), and it may be that biofilm formation by *B. subtilis* is needed to prevent *C. rodentium* pathology by better excluding the pathogen through competition. However, pre-treatment of mice with *B. subtilis* PY79 only mildly reduced the number of *C. rodentium* CFU in the colon in the original study (D'Arienzo et al., 2006), and no difference in *C. rodentium* CFU or any alteration in colonization in *B. subtilis* NCIB 3610-treated mice was observed in the

studies by Jones and colleagues (Jones and Knight, 2012; Jones et al., 2014), indicating that the assumption that *B. subtilis* protects through exclusion of *C. rodentium* from the GI tract may not be correct. It should be noted that a few key differences between studies by the two groups could explain the differences observed. One is that *B. subtilis* PY79 is a domesticated laboratory strain that does not form robust biofilms as *B. subtilis* NCIB 3610 does under *in vitro* conditions (Branda et al., 2001), which may affect the way *B. subtilis* colonizes the GI tract. Another is that D'Arienzo and colleagues infected suckling NIH Swiss mice with  $10^3$  CFU *C. rodentium* while Jones and Knight used C57BL/6J mice infected with  $5 \times 10^8$  CFU *C. rodentium*, so perhaps with the increased pathogen inoculum, *B. subtilis* was not able to compete against *C. rodentium* as well in the study by Jones and Knight. Also, the sIgA repertoire is known to be different between different strains of mice, which leads to differences in microbial composition (Fransen et al., 2015) that could affect how *B. subtilis* and *C. rodentium* interact within the GI tract. The other possibility is that *B. subtilis* exerts its protective effect through modulation of host processes through EPS. Jones and colleagues tested if *B. subtilis* reinforces host barrier integrity during *C. rodentium* infection, which would reduce pathogen translocation into host tissues and reduce triggering of inflammatory responses that drive disease. However, *B. subtilis*-treated mice did not have reduced gut leakiness during *C. rodentium*-infection, as measured by the translocation of orally administered fluorescein isothiocyanate (FITC)-labeled dextran into serum (Jones et al., 2014), indicating that *B. subtilis* did not alter host barrier functions. The pathology of *C. rodentium*-induced colitis depends on CD4<sup>+</sup> T cells recruited to the GI tract, which mediate pro-inflammatory responses that drive IBD-like pathologies such as crypt hyperplasia, loss of goblet cells, and increased pro-inflammatory cytokine levels

(Higgins et al., 1999). When assessing *C. rodentium*-induced disease in *B. subtilis*-treated mice, it was observed that *B. subtilis* reduced crypt heights and levels of pro-inflammatory cytokines and that the goblet cells were left intact (Jones and Knight, 2012; Jones et al., 2014), suggesting that *B. subtilis* protects hosts by altering host inflammatory processes. Jones and colleagues hypothesized that *B. subtilis* EPS might have immunomodulatory properties that mediate protection, and indeed, systemic administration of purified EPS from *B. subtilis* recapitulated protection from *C. rodentium*-induced colitis by *B. subtilis* spores (Jones et al., 2014). This suggested that *B. subtilis* exerts protection by producing EPS, an immunomodulatory polysaccharide that prevents inflammation. (Jones et al., 2014).

Host recognition of microbial molecules generally starts with PRRs. Studies of other probiotics that work through immunomodulation found that signaling through TLR2 was required for their beneficial effects (Danne et al., 2017; Jeon et al., 2012; Round and Mazmanian, 2010). Based on this, it was hypothesized that EPS also alters the immune system by first engaging a PRR. Indeed, EPS failed to protect TLR4-deficient mice from *C. rodentium*-induced colitis as well as mice deficient in myeloid differentiation primary response protein 88 (MyD88), the adaptor protein critical for multiple TLR signaling pathways (Jones et al., 2014), indicating that EPS exerts its effects through TLR4 signaling. TLR4 is best characterized as the mediator of signaling induced by lipopolysaccharide (LPS), a microbial ligand found mostly on Gram-negative bacteria (Hoshino et al., 1999). However, the process by which the signal initiates is quite complex. Upon release from the microbe, LPS is first bound to LPS-binding protein, an acute phase protein in the serum (Tobias et al., 1986). LPS is then delivered to CD14, a glycosylphosphatidylinositol (GPI)-anchored glycoprotein found on the

surface of MNPs (Hailman et al., 1994). Originally, CD14 was thought to be crucial for the recognition of LPS since CD14-deficient mice were insensitive to lethal challenge with LPS and cells from these mice failed to produce pro-inflammatory cytokines TNF- $\alpha$  and IL-6 in response to LPS, though limited production could be detected at high LPS concentrations (Haziot et al., 1996). However, CD14 does not mediate intracellular signaling, and further studies suggested that CD14 brings LPS to TLR4 where signaling is initiated. Transfer of LPS from CD14 to TLR4 was recently confirmed (Ryu et al., 2017). Myeloid differentiation factor 2 (MD-2) is the major protein that binds LPS when complexed with TLR4 (Akashi et al., 2003), and this complex initiates signaling through the intracellular Toll-IL-1 receptor homology (TIR) domains of TLR4 (Poltorak et al., 1998). The signal is amplified by the adaptor protein, MyD88, and leads to downstream activation of the transcription factor nuclear factor kappa light chain enhancer of activated B cells (NF- $\kappa$ B), which drives gene expression of pro-inflammatory cytokines (Kawai et al., 1999). It is not clear how TLR4 signaling contributes to EPS-induced protection from *C. rodentium*-induced colitis, given that the activation of TLR4 is generally associated with pro-inflammatory responses while EPS decreased inflammation during disease. Nonetheless, understanding both *in vitro* and *in vivo* pathways of EPS-induced activation of TLR4 signaling, and the resulting host responses will be key to understanding the therapeutic potential of *B. subtilis* EPS.

### **Anti-Inflammatory Effect of *Bacillus subtilis* Exopolysaccharide**

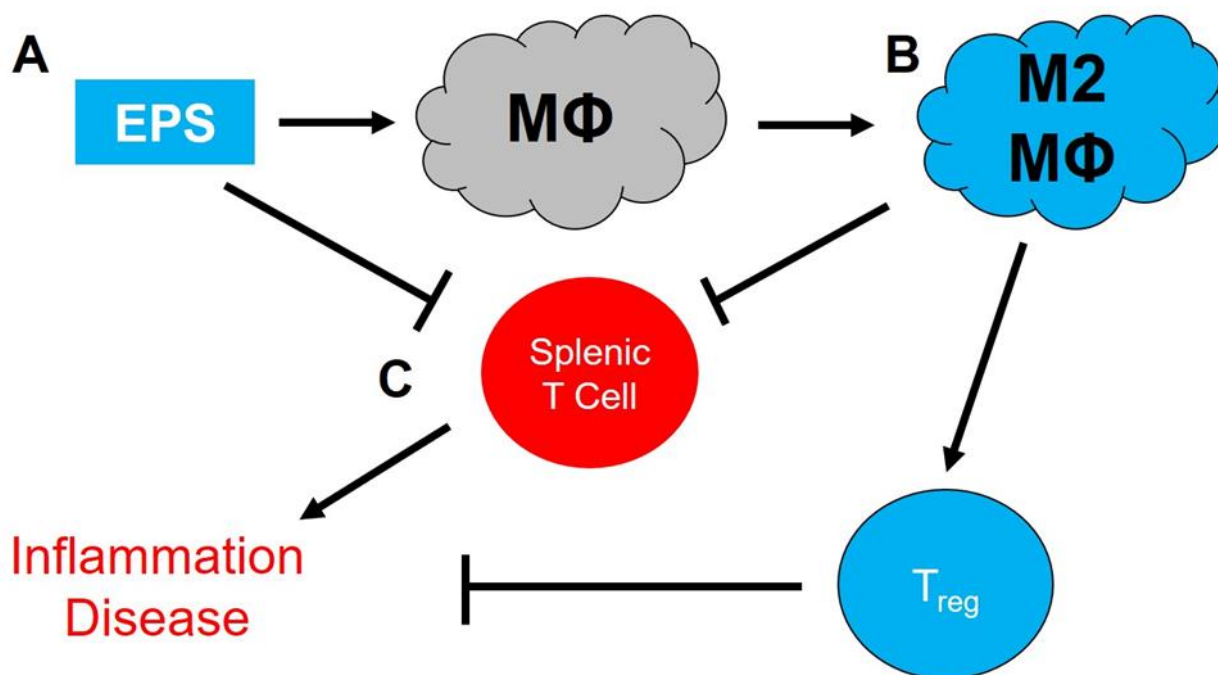
Intraperitoneal (i.p.) administration of EPS is sufficient to confer protection in murine *C. rodentium*-induced colitis (Jones et al., 2014). Under homeostatic conditions, the peritoneal cavity myeloid compartment is dominated by M $\Phi$ s. There are two subsets of peritoneal M $\Phi$ : large peritoneal M $\Phi$ s (LPM, defined by CD11b<sup>high</sup>F4/80<sup>high</sup>) that



represent the majority, and small peritoneal MΦs (SPM, defined by CD11b<sup>+</sup>F4/80<sup>+</sup>) that represent the minority (Ghosn et al., 2010). Upon stimulation with i.p. LPS injection, the peritoneal cavity becomes dominated instead by SPMs, which show enhanced antimicrobial nitric oxide (NO) production compared to the LPM counterparts (Ghosn et al., 2010). LPMs are thought to be the long-term residents of the peritoneal cavity but disappear after i.p. LPS injection; LPMs are hypothesized to be recruited to different nearby tissues for various purposes. For example, it was recently demonstrated that during sterile liver injury, LPMs, defined by their unique expression of GATA-binding factor 6 (GATA-6), were recruited directly from the peritoneal cavity to the liver surface through the mesothelium to mediate wound healing (Wang and Kubes, 2016). Since LPMs are the major innate immune cell within the peritoneal cavity and MΦs are well known to sense microbial products to mediate various functions, EPS was hypothesized to alter the functions of LPMs to exert its protective functions. Indeed, EPS failed to protect mice from *C. rodentium* infection when mice were depleted of peritoneal MΦs through the administration of clodronate-loaded liposomes, indicating that MΦs are required for protection by EPS (Paynich et al., 2017). MΦs are described in a spectrum between two polarization states ranging from pro-inflammatory to anti-inflammatory functions. Since EPS reduces inflammation during *C. rodentium* infection that is primarily thought to be driven by T cells (Higgins et al., 1999), EPS was proposed to drive an anti-inflammatory polarization of MΦs that limit inflammation during *C. rodentium*-induced colitis. Paynich and colleagues compared the CD11b<sup>high</sup>F4/80<sup>high</sup> LPM compartment between phosphate buffered saline solution (PBS)- and EPS-treated mice 3 days after i.p. injection and found that the LPMs resembled M2 MΦs in EPS-treated mice, showing increased expression of M2 markers, including CD206 and Arg-1

(Paynich et al., 2017). These EPS-induced MΦs limited splenic T cell activation *in vitro*, suggesting that EPS induces anti-inflammatory M2 MΦs that suppress T cell functions (**Figure 1A**) (Paynich et al., 2017).

Although M2 MΦs can inhibit T cell activation by depletion of arginine through



**Figure 1: Established Model for the Anti-Inflammatory Effect of *B. subtilis* EPS.** **A.** EPS drives anti-inflammatory M2 polarization of MΦs. **B.** EPS-induced M2 MΦs limit T cell activation and promote T<sub>reg</sub> differentiation. **C.** EPS limits systemic T cell activation, limiting disease due to inflammation. Adapted from Paynich and colleagues, *Journal of Immunology*, 2017.

the action of Arg-1 expressed by EPS-induced M2 MΦs, supplementation of L-arginine did not restore T cell activation in co-cultures with EPS-induced M2 MΦs. However, neutralization of the inhibitory cytokine TGF-β and blockade of the inhibitory cell surface molecule programmed death-ligand 1 (PD-L1) using antibodies *in vitro* restored T cell activation, suggesting that EPS-induced M2 MΦs inhibit T cells through TGF-β and PD-L1 (Paynich et al., 2017). In addition, co-cultures of EPS-induced M2 MΦs with splenocytes resulted in an increase in CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells, and there was an

increased percentage of Foxp3<sup>+</sup> T cells in the peritoneal cavity of EPS-treated mice *in vivo*, which suggests that EPS-induced M2 MΦs promote T<sub>reg</sub> cell differentiation (**Figure 1B**) (Paynich et al., 2017). Also, cells from the mesenteric lymph nodes (mLN) of EPS-treated mice were hyporesponsive to stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin, indicating that EPS limits T cell functions *in vivo* (**Figure 1C**) (Paynich et al., 2017). Finally, transfer of these EPS-induced MΦs to recipient mice was sufficient for protection from *C. rodentium*-induced colitis (Paynich et al., 2017), indicating that *B. subtilis* EPS protects hosts from colitis in large part through these anti-inflammatory M2 MΦs that modulate T cell activation during disease *in vivo*, limiting inflammation and improving disease outcomes.

Administration of EPS results in anti-inflammatory M2 MΦs in the peritoneal cavity, but it is not known if EPS has similar effects in other tissues; after all, i.p. injection is considered a systemic route of administration (Turner et al., 2011). EPS-treated mice had reduced levels of T cell effector cytokines in the serum after *in vivo* administration of anti-CD3ε antibodies that activate T cells (Paynich et al., 2017), indicating that i.p. administration of EPS affects T cells systemically. Consistent with this view, orally administered *B. subtilis* spores, but not the *epsH* mutant spores, prevented allergic inflammation in the lung in a house dust mite-induced eosinophilic inflammation model (Swartzendruber et al., 2019), indicating that the anti-inflammatory effect of EPS is systemic, and can reduce inflammation in tissues beyond the GI tract. To date, the structure and composition of EPS remain unknown.

In summary, *B. subtilis* is a probiotic bacterium that produces EPS, a component of its biofilm that has systemic anti-inflammatory properties. *B. subtilis* has been consumed by humans for many years and is not known to cause disease in

immunocompetent individuals. Additionally, it can be easily administered as durable spores by the oral route, and there is a potential to use purified EPS as a pharmacologic agent. This anti-inflammatory property of *B. subtilis* is likely to benefit many patients suffering from various diseases with inflammatory pathophysiology, and to this end, the specific mechanisms by which EPS modulates host responses in other inflammatory disease conditions remain to be elucidated. Here, we present our study on exploring the potential to use EPS for one such disease with a systemic inflammatory pathology, sepsis, one of the most severe manifestations of systemic infection.

### **Sepsis and *Staphylococcus aureus***

#### **Sepsis Epidemiology and Pathophysiology**

Sepsis is defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection (Singer et al., 2016). Sepsis is one of the most severe manifestations of ongoing infection with mortality rates approaching 50% in some cases (Angus et al., 2001). Sepsis accounted for about 6% of all US adult hospitalizations in 2014, estimated to be over a million cases a year (Rhee et al., 2017). While controversial, sepsis is considered the most expensive condition to manage in the US (Torio and Andrews, 2006). Sepsis is generally thought to start when microbial ligands engage host immune cells, setting off a pro-inflammatory response designed to clear microbial pathogens: activation of innate immune cells, production of cytokines and chemokines to recruit and activate additional immune cells, and activation of complement cascades to further support pro-inflammatory responses. While many processes are thought to contribute to the pathophysiology of sepsis, the most clinically concerning is the loss of endothelial integrity. Recruitment of additional immune cells to tissues generally occur from the circulation. Initially, the local pro-inflammatory

mediators activate the nearby endothelial cells and induce their expression of leukocyte adhesion molecules such as vascular cell adhesion protein 1 (VCAM-1) (Bevilacqua et al., 1987; Osborn et al., 1989). Circulating immune cells are captured by leukocyte adhesion molecules and start rolling along the endothelial surface until activated by chemokines through GPCRs (Atherton and Born, 1972; Kim et al., 2003). This leads to the expression of integrins such as intercellular adhesion molecule 1 (ICAM-1) on endothelial cells that mediate adhesion between immune cells and the endothelium (Dustin and Springer, 1988; Dustin et al., 1986). Finally, the adhered immune cell crawls to a junction between endothelial cells (Phillipson et al., 2006) where it squeezes through to the basolateral side to enter the tissue, a process defined as diapedesis. This process is accommodated in part by the loosening of the endothelial junctions by the increase in intracellular free calcium ions in response to cross-linking of adhesion molecules, which activates myosin light chain kinases and subsequent actin-myosin fiber contractions (Hixenbaugh et al., 1997; Huang et al., 1993). This results in a small separation of endothelial cells that immune cells can squeeze through, which increases vascular permeability. Increased leakiness of the endothelium is generally maintained locally, but under severe systemic conditions such as sepsis, vascular permeability is increased systemically, and intravascular fluid is lost into the tissues. This makes maintaining blood pressure difficult, and perfusion of organs can become limited due to cardiovascular collapse, leading to multi-organ failure. Another major complication during sepsis that stems from endothelial dysfunction is related to the coagulation system. Coagulation is mediated by a complex cascade involving plasma coagulation factors and platelets, designed for deposition of fibrin at sites of tissue damage to prevent leakage of blood contents. Many aspects of inflammation are known to activate

the coagulation cascade, but the most prominent way sepsis is thought to initiate the coagulation cascade is the tissue factor that is produced as a result of tissue and endothelial damage (Osterud and Rapaport, 1977). It should be noted that microbial ligands like LPS have been shown to directly activate platelets as well (Andonegui et al., 2005), though the contribution of such activation to sepsis is not clear. Due to the systemic nature of the disease, sepsis can initiate widespread activation of coagulation cascades within the vasculature, a condition known as disseminated intravascular coagulation (DIC). DIC is thought to precipitate two major issues: widespread microvascular thrombosis that blocks blood flow resulting in organ dysfunctions, and rapid depletion of platelets that further complicates endothelial dysfunction and maintenance of blood pressure for organ perfusion (Akca et al., 2002; Toh and Alhamdi, 2013). DIC is a common complication of severe sepsis and is often associated with mortality (Ogura et al., 2014). Despite extensive clinical studies, sepsis remains an often lethal challenge that is difficult for physicians to identify, diagnose, and manage.

Conditon	Evaluation	Criteria for Action
Suspected sepsis	Quick SOFA (qSOFA)	If qSOFA $\geq 2$ , evaluate for sepsis
Sepsis	SOFA	Diagnose sepsis if SOFA $\geq 2$
Septic Shock	Mean arterial pressure (MAP), serum lactate level	If vasopressors required to maintain MAP $\geq 60$ mmHg and serum [lactate] $\geq 2$ mM, diagnose septic shock

**Table 3: Evaluation of Sepsis in Patients.** As recommended by Singer and colleagues, *Journal of the American Medical Association*, 2016.

Clinically, patients suspected of sepsis are evaluated using a sepsis-related organ failure assessment (SOFA) score. SOFA score system evaluates functions of major organ systems, determining a score for estimated organ damage (Singer et al., 2016). For example, mean arterial pressure (MAP) greater than or equal to 70 mmHg, considered relatively normal in terms of overall cardiovascular function, is given a score of 0 while MAP less than 70 mmHg would be given a score of 1. Higher scores would be given if pharmacologic agents had to be used to maintain MAP. Functions of other systems such as renal, hepatic, respiratory, and coagulation systems are evaluated and given scores, and a cumulative score is determined to diagnose sepsis, along with microbiologic evidence of infection. Increased serum lactate levels in patients, a sign of metabolic acidosis due to severe illness, with hypotension resistant to fluid resuscitation has been associated with much higher mortality rates, so these criteria are used additionally to diagnose septic shock (Singer et al., 2016). Since many patients often present with vague symptoms, and since sepsis is a rapidly developing condition, physicians are encouraged to use a broad screening tools such as quick SOFA (qSOFA) scoring, a much simpler version of the SOFA score, to identify suspected patients and subsequently evaluate for sepsis, as outlined in **Table 3**. However, many patients with modest dysfunctions according to SOFA scores can rapidly deteriorate, and the diagnostic criteria for sepsis remain controversial (Singer et al., 2016), making the diagnosis of sepsis a challenge. Treatment of sepsis patients focuses on two primary approaches.

The best highlighted is antimicrobial therapy to reduce inflammatory stimuli. The other is to limit host responses, especially on maintaining MAP and preventing platelet depletion. Due to high mortality rates, sepsis patients are often aggressively treated: broad-spectrum antimicrobial therapy initiated within one hour, aggressive

testing to identify the infectious source, immediate intravascular fluid resuscitation, vasopressors, and more invasive interventions such as blood or platelet transfusions and mechanical ventilation if necessary (Rhodes et al., 2017). Despite aggressive management, mortality rates for sepsis remain high (Angus et al., 2001; Rhee et al., 2017), highlighting a critical need for novel strategies to manage and treat sepsis patients. For a majority of sepsis cases, the causative organism is not identified, though some of this is presumably attributed to early initiation of broad-spectrum antimicrobial therapy that could impede detection of organisms through traditional culture methods (Vincent et al., 2009). Of the cases for which the causative organism is documented, *S. aureus* remains the top etiologic agent of sepsis, accounting for about 20% of all cases (Vincent et al., 2009).

### **Clinical Considerations for *Staphylococcus aureus* Infections**

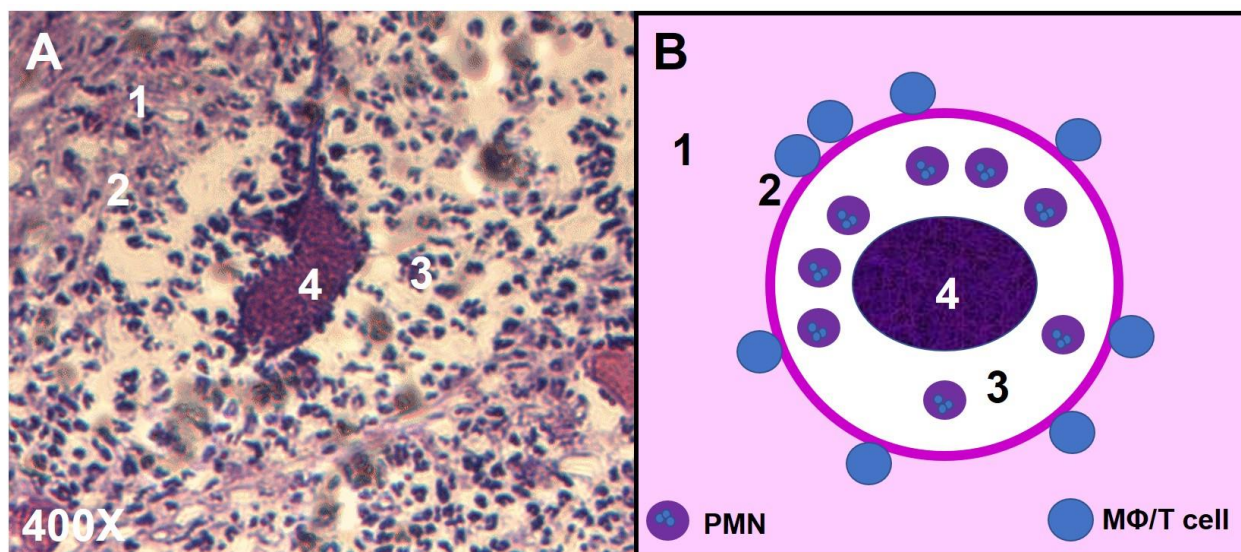
*S. aureus* is a gram positive bacterium found on the skin and anterior nares of colonized individuals. About 30% of the general population is thought to be colonized with *S. aureus* without apparent disease, and *S. aureus* is considered a part of the healthy microbiota of the skin (Gorwitz et al., 2008). However, upon breaching epithelial barriers, *S. aureus* can seed into underlying soft tissue and cause disease generally described by a cutaneous abscess; *S. aureus* is the primary cause of skin and soft tissue infections (SSTI), estimated to be about 3.4 million emergency visits in 2005 (Pallin et al., 2008). While SSTIs are the most common form of *S. aureus* infection, *S. aureus* can penetrate into deeper tissues and cause severe diseases including necrotizing fasciitis, necrotizing pneumonitis, osteoarthritic infections, infective endocarditis, and bacteremia, to name a few.



Like many infectious diseases, *S. aureus* bloodstream infections are aggressively managed with antibiotics designed to reduce the inflammatory stimuli (Liu et al., 2011). Historically, penicillin was introduced in the 1940s, that successfully reduced case fatality rates due to *S. aureus* bacteremia (Blair and Carr, 1960; Fleming, 1929). However, penicillin resistance was reported only a few years later, and within a decade, antibiotic-resistant *S. aureus*, harboring a plasmid-encoded penicillinase, became widely prevalent (Blair and Carr, 1960). A penicillinase-resistant  $\beta$ -lactam antibiotic, methicillin, was introduced in the late 1950s to combat these resistant *S. aureus* strains, but the medical community was met with methicillin-resistant *S. aureus* (MRSA) by 1961 (Barber, 1961), which limited treatment efficacy. Antibiotic resistance continues to be a significant problem today, where strains resistant to a variety of standard antimicrobial agents are reported, including vancomycin-resistant strains (Hiramatsu et al., 1997; Liu et al., 2011; Weigel et al., 2003), making treatment of *S. aureus* infections a challenge. Several novel antimicrobial agents are in the development pipeline (Holubar et al., 2016), but resistance can and will likely arise as resistance is often conferred through mobile genetic elements that are easily acquired, and that will perpetuate the process of developing antimicrobials and resistances. Therefore, novel strategies to combat *S. aureus* infections are critically needed. A major goal of antimicrobial therapy during sepsis is to reduce the inflammatory stimuli by targeting the pathogens for removal. However, an alternative approach may be to target the host to limit overt inflammation and promote survival. To this end, understanding host responses against *S. aureus* during bloodstream infection is critical.

## Host Responses to *Staphylococcus aureus* Infection

As in other bacterial infections, the initial host response against *S. aureus* is induced through recognition of microbial molecules through PRRs: for *S. aureus*, recognition of lipopeptides through TLR2 is the primary sensor mechanism (Hashimoto et al., 2006; Takeuchi et al., 2000).



**Figure 2: Histology of *S. aureus*-Induced Murine Renal Abscess.** A female C57BL/6J mouse was systemically infected with *S. aureus* USA300 (LAC) and kidneys were assessed 2 days post infection (d.p.i) for histopathologic features. **A.** Hematoxylin and eosin (H&E) stained section of a *S. aureus* renal abscess. 400x magnification. **B.** Schematic representation of histopathologic features of the *S. aureus* renal abscess 1. Surrounding renal tissue. 2. Fibrous capsule surrounded by additional immune cells like macrophages and T cells. 3. Damaged tissue with necrotic cells and neutrophils. 4. Infected focus of *S. aureus*. Image by Paik and colleagues.

This recognition results in the production of signals and chemokine gradients to recruit neutrophils to the site of infection, which is critical for initial containment of the pathogen. Depletion of neutrophils results in increased mortality in an animal model of *S. aureus* bacteremia (Alonzo III et al., 2012), and patients with defective neutrophil functions are predisposed to infections, predominantly *S. aureus* (Marciano et al., 2015). Once at the site of infection, neutrophils phagocytose *S. aureus* and try to kill the

pathogen through a complex intracellular process involving endosomal acidification, production of RNS and ROS, and release of AMPs. In addition, neutrophils can release DNA and toxic granules known as neutrophil extracellular traps as they undergo cell death (NETosis) which traps bacteria for killing (Brinkmann et al., 2004). *S. aureus* harbors many mechanisms to withstand the actions of neutrophils, requiring additional host mechanisms for immunity such as MΦs and T cells. Monocytes, which differentiate into MΦs within tissues, are generally the first cell type recruited after neutrophils to augment anti-bacterial immunity (Volkman and Gowans, 1965). MΦs use similar mechanisms as neutrophils to contain pathogens but have the additional capacity to present antigens and coordinate subsequent immune responses mediated by the adaptive arm of immunity. T cells of the adaptive immune system can then augment bacterial killing through the production of pro-inflammatory cytokines, which help recruit more neutrophils and monocytes and increase their killing capacities (Held et al., 1999). These cells typically constitute the classical pathological feature of *S. aureus* infections characterized as abscesses. Abscesses form as neutrophils arrive at the infected site and employ its antimicrobial defenses. *S. aureus* withstands neutrophils while tissue damage occurs, creating a core containing bacteria, necrotic debris, and neutrophils. The additional immune cells recruited are thought to contain the infection by promoting the development of the fibrous capsule at the edge of the infected foci, forming a mature abscess structure, as outlined in **Figure 2**.

### ***Staphylococcus aureus* Evasion of Host Immunity: Recognition**

Unfortunately, *S. aureus* has numerous mechanisms to interfere with the host immune response at multiple levels, as described in **Table 4**. For one, *S. aureus* can interfere with host recognition, mainly by counteracting TLR2 signaling. One way *S.*

*aureus* does this is through a lipoylated E2 subunit of the pyruvate dehydrogenase (E2-PDH), an essential metabolic enzyme complex, which is also secreted.

Evasion strategy	<i>S. aureus</i> factor	Host target	Mechanism
TLR2 recognition	Lipoylated E2-PDH, SSL3	TLR2 on various cells	Reduced immune activation, reduced signaling
Immune cell chemotaxis	CHIPS, Staphopain A	Chemokine receptors	Block chemokine signaling, cleave CXCR2
Limit opsonophagocytosis	Protein A, SCIN	Immunoglobulins, complement	Bind antibodies and block opsonic activity, inhibit complement activation
Resist phagocyte killing	Staphyloxanthin, SodA/M	ROS, O <sub>2</sub> <sup>-</sup>	Antioxidant activity, inactivate superoxides
Kill immune cells	Pore-forming toxins, SE and SEI, TSST-1 Protein A	Specific receptors, TCR-MHC, BCR	Form pores on cell membrane, activation-induced cell death

**Table 4: Major Mechanisms of Immune Evasion by *S. aureus*.**

The lipoylated protein has structural similarities with TLR1-2 agonists and is thought to limit TLR2 signaling through direct antagonism, interfering with TLR2 signaling induced by *S. aureus* lipopeptides, resulting in limited host response *in vivo* and promoting bacterial survival (Grayczyk et al., 2017). *S. aureus* can also do this by Staphylococcal superantigen-like protein 3 (SSL3), which binds to TLR2 and antagonizes signaling induced by TLR2 agonists in cells *in vitro* (Bardoel et al., 2012). By limiting activation by TLR2, *S. aureus* evades recognition by the host to promote bacterial survival. Another mechanism by which *S. aureus* can subvert host responses is by impairing immune cell recruitment, that depends on immune cell recognition of chemokine ligands through chemokine receptors. Chemotaxis inhibitory protein of *S. aureus* (CHIPS) is a protein secreted by *S. aureus* that binds to phagocytes to interfere with chemokine-induced migration (Haas et al., 2004). Similarly, Staphopain A is a protease that cleaves C-X-C motif chemokine receptor 2 (CXCR2) on the surface of

neutrophils, limiting neutrophil homing to infective tissues. (Laarman et al., 2012). Through these mechanisms, *S. aureus* limits immune cell recruitment to infected sites, promoting bacterial survival.

### ***Staphylococcus aureus* Evasion of Immunity: Withstanding the Killing**

Once the host recognizes pathogens and recruits MNPs to the infected site, bacterial clearance is thought to occur through phagocytosis of bacteria for intracellular killing. One mechanism by which the host enhances phagocytosis of pathogens is through antibody- and complement-mediated opsonization. Opsonization is the process by which antibodies and complement deposit on bacterial surfaces, enhancing uptake by phagocytes that recognize the deposited antibodies or complement through antibody fragment crystallizable region (F<sub>c</sub>) receptors and complement receptors, respectively (Caron and Hall, 1998). Protein A is a molecule produced by *S. aureus* that binds immunoglobulins and blocks their opsonic activity (Forsgren and Quie, 1974), which would reduce F<sub>c</sub> receptor-mediated phagocytosis of bacteria. On the side of complement, Staphylococcal complement inhibitor (SCIN) is a C3 convertase inhibitor that prevents complement activation and subsequent deposition on bacterial surfaces, limiting opsonization and uptake by human neutrophils (Roijakkers et al., 2005). Even when *S. aureus* is taken up by phagocytes, *S. aureus* harbors mechanisms to resist phagocyte-mediated killing. Staphyloxanthin is a carotenoid pigment that gives *S. aureus* the hallmark golden color, but it is also an antioxidant that allows *S. aureus* to withstand neutrophil-mediated killing through the production of RNS and ROS (Liu et al., 2005). *S. aureus* also encodes two superoxide dismutases, SodA and SodM, which inactivate superoxide radicals to resist phagocyte-mediated killing (Karavolos et al., 2003).

Through these mechanisms, *S. aureus* subverts the host phagocytic responses, promoting its persistence within hosts.

### ***Staphylococcus aureus* Evasion of Immunity: Killing Immune Cells**

*S. aureus* also harbors more sinister mechanisms to counteract the host immune response, a deleterious challenge to the immune system through toxins. *S. aureus* produces seven different pore-forming toxins, some of which target immune cells. These leucocidins bind to a specific host cell receptor and subsequently recruit another subunit for oligomerization on the cell surface, forming a pore through the host cell membrane and killing it (Finck-Barbançon et al., 1993; Noda et al., 1982; Woodin and Wieneke, 1963; 1967). The most notorious leucocidin is Panton-Valentine leucocidin (PVL), which recognizes human complement C5a receptor (C5aR) to kill neutrophils (Spaan et al., 2013). Neutrophils are not the only cells to be targeted by leucocidins, as leucocidin ED (LukED) had been demonstrated to have a broad repertoire of cells it kills by targeting C-C motif chemokine receptor 5 (CCR5), CXCR1, and CXCR2 on T cells, NK cells, neutrophils, monocytes, and DCs (Alonzo III et al., 2012a; 2012b; Reyes-Robles et al., 2013). In the context of abscesses, monocytes are additionally recruited to reinforce neutrophil-mediated antimicrobial defense, but *S. aureus* also harbors ways to target MΦs for killing. A study demonstrated that *S. aureus* could produce deoxyadenosine from extracellular DNA released by neutrophils during NETosis to kill U937 cells, a MΦ cell line (Thammavongsa et al., 2013; Winstel et al., 2018). *S. aureus* mutants deficient in nuclease and adenosine synthase, *nuc* and *adsA* respectively, failed to exclude F4/80<sup>+</sup> MΦs from the core of renal abscesses (Thammavongsa et al., 2013), suggesting that *S. aureus* utilizes DNA released by neutrophils through NETosis to kill MΦs, promoting bacterial survival within abscesses. It should be noted that the killing of U937 MΦs by *S.*

*aureus*-produced deoxyadenosine was very mild, and that stressing the DNA processing system using pentostatin, a purine analog, was required for robust killing of MΦs. It is not clear if such stress would occur *in vivo*, but *S. aureus* mutants incapable of producing deoxyadenosine could not exclude MΦs from the abscesses, indicating that the killing of MΦs likely occurs *in vivo*. By targeting multiple immune cells for killing, *S. aureus* is able to evade a variety of host responses to establish itself within various host tissues.

### ***Staphylococcus aureus* Evasion of Immunity: Superantigens**

The cells of the adaptive immune system can additionally be targeted for killing through activation-induced cell death, which is mediated by superantigens (SAg) produced by *S. aureus*. Staphylococcal enterotoxins and enterotoxin-like proteins (SE and SEI) and related SAg are secreted proteins that bind to both the V $\beta$  region of the T cell receptor (TCR) complex and MHC class II on antigen presenting cells, activating up to 20% of the T cell repertoire (Fraser, 1989; Peavy et al., 1970; White et al., 1989). This polyclonal activation of T cells results in massive production of pro-inflammatory cytokines, which can cause a phenomenon called cytokine storm that causes toxic shock syndrome, an often fatal complication (Todd et al., 1978). Concurrently, the inappropriately activated T cells can impede proper adaptive immune responses, either by activation-induced cell death that removes specific T cells from the repertoire and by limiting activation of T cells through presented antigens (White et al., 1989; Xu et al., 2014; Ziegler et al., 2011). Similarly, the other arm of adaptive immunity can be targeted by protein A which has SAg activity against B cells that results in apoptotic removal of B cells from the repertoire, limiting antibody responses (Goodyear and Silverman, 2004).

By targeting different immune cells for destruction, *S. aureus* is able to impede immune effector cell functions directly, promoting its survival.

### **Concluding Remarks**

During infection, *S. aureus* utilizes many mechanisms to perturb the immune response at multiple levels, resulting in a suboptimal host response not sufficient for bacterial clearance. This allows the pathogen to persist and, in the case of systemic infection, overt systemic inflammation occurs as a prolonged attempt to clear the pathogen. This perturbed inflammation can often result in the manifestation of sepsis and lethality, despite aggressive antimicrobial therapy to reduce bacterial burden. Therefore, an optimal approach to managing patients with systemic *S. aureus* infections is to bolster host antimicrobial defenses to reduce the bacterial burden while limiting inflammation to reduce the tissue damage and prevent the development of lethal complications such as sepsis. *B. subtilis* EPS is a probiotic-derived agent with potentially systemic anti-inflammatory properties. We reasoned that this anti-inflammatory agent could be used to limit inflammation during *S. aureus* bloodstream infections, reducing the host burden of disease. Herein, we focused our studies on tests of the protective potential of EPS against *S. aureus* bloodstream infection using a murine model, and to elucidate the specific mechanisms by which protection occurs, with the hope that these insights will help develop novel strategies to manage sepsis and reduce patient suffering.



## CHAPTER TWO

### MATERIALS AND METHODS

#### Mice

All studies utilizing animals were reviewed and approved by the Institutional Animal Care and Use Committee at Loyola University Chicago Health Sciences Division (Maywood, IL). Unless otherwise specified, all mice were bred in house at the Comparative Medicine Facility at Loyola or purchased from Jackson Laboratories (Bar Harbor, ME). ND4 Swiss Webster mice were purchased from Envigo (Indianapolis, IN). Founder mice for in house breeding were purchased from Jackson Laboratories. All mice were housed at the Comparative Medicine Facility at Loyola and fed standard sterile chow. All mice used for this study is listed in **Table 5**. Unless otherwise noted, C57BL/6J mice were used.

Mice	Genotype	Source
C57BL/6J	Wild-type/Inbred	In House/Jackson Laboratories
BALB/cJ	Wild-type/Inbred	In House/Jackson Laboratories
ND4 Swiss Webster	Wild-type/Outbred	Envigo
TLR4 KO	B6.(Cg)- <i>Tlr4</i> <sup>tm1.2Karp</sup> /J	In House/Jackson Laboratories
MyD88 KO	B6.129P2(SJL)- <i>Myd88</i> <sup>tm1.1Defr</sup> /J	In House/Jackson Laboratories

**Table 5: Mice Used in This Study.**

### Common Reagents

All bacteriologic media were purchased from BD Biosciences (San Jose, CA). All cell culture media components were purchased from Thermo Fischer Scientific (Waltham, MA). Fetal bovine sera (FBS) for cell culture were purchased from Atlanta Biologicals (Flowery Ranch, GA). Modified Roswell Park Memorial Institute (RPMI) medium was used for all cell culture experiments (RPMI base medium supplemented with 2.4 mM L-glutamine, 1x minimal essential medium (MEM) vitamins solution, 50 U/mL penicillin, 50 µg/mL streptomycin, 0.5 µg/mL amphotericin B, 0.5x non-essential amino acids, 0.5x essential amino acids, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mM sodium pyruvate, 0.195 % sodium bicarbonate (NaHCO<sub>3</sub>), 30 µg/mL gentamicin, 3.5 x 10<sup>-4</sup> % 2-mercaptoethanol, 10% heat-inactivated FBS). For antibiotic-free RPMI medium, penicillin, streptomycin, and gentamycin were excluded. All antibodies were purchased from BioLegend (San Diego, CA) unless otherwise specified. All chemical reagents were purchased from MilliporeSigma (Burlington, MA) unless otherwise specified.

### Preparation of *B. subtilis*-Derived EPS

EPS was isolated from culture supernatants of *B. subtilis* DS991 (overproduces and secretes EPS) or DK4623 (DS991 derivative with lytic genes (Wang et al., 2014) deleted) grown to stationary phase in 1% tryptone phosphate broth (1% tryptone, 25 mM phosphate, 0.1 M sodium chloride (NaCl)) or minimal salts glutamate glycerol (Msgg) medium. Alternatively, EPS was prepared from *B. subtilis* DK6251 (Isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible *eps* operon with gamma-polyglutamic acid (γ-PGA) deficiency) grown as a lawn on Luria Bertani (LB, Miller formulation) agar (1.5%) plates with 1 mM IPTG. For ΔEPS, *B. subtilis* DS5187 (DS991 derivative that cannot

make EPS) was used. All bacterial strains used in this study are listed in **Table 6**. EPS was extracted by precipitation with 75% ethanol at -20°C. The precipitate was pelleted by centrifugation at 13,700 x g at 4°C for 30 min, resuspended in 0.1 M pH 8 Tris buffer, and treated with 67 µg/mL DNase and 330 µg/mL RNase at 37°C for 2 hours (hr), followed by digestion with 40 µg/mL proteinase K at 55°C for 2 hr. EPS was then purified on diethylaminoethyl (DEAE)-cellulose (GE Healthcare, Chicago, IL) ion exchange column chromatography or gel filtration on Sephacryl S-500 (GE Healthcare). Carbohydrate-positive fractions were identified, and total carbohydrate content was estimated using a modified phenol sulfuric acid assay (Albalasmeh et al., 2013; Masuko et al., 2005). All EPS preparations were assessed for the lack of protein and nucleic acid content by spectrometry and also for their ability to induce peritoneal M2 MΦ as previously described (Paynich et al., 2017) prior to use. All three strains of WT mice in this study were capable of generating peritoneal M2 MΦs in response to i.p. EPS administration as described previously (Paynich et al., 2017).

### **Preparation of *S. aureus* Cultures**

Starter cultures of all *S. aureus* strains were prepared in tryptic soy broth (TSB) medium, cultured at 37°C overnight. *S. aureus* LAC and AH1263 (plasmid-cured derivative of LAC (Boles et al., 2010)) were used in many studies interchangeably, but we did not observe noticeable differences in outcomes. Transposon (Tn) insertion mutant library strains from the University of Nebraska Transposon Mutant Library (Fey et al., 2013) were used for SAg studies. For bloodstream infection experiments, starter cultures were freshly diluted 1:100 into TSB and cultured at 37°C to exponential phase for 3 hr. Inoculum was prepared by washing the subculture three times in 10 mL PBS, normalized to an inoculum of 10<sup>8</sup> CFU/mL in PBS on the day of infection using optical

density (OD) measurements on Genesys 10S UV-Vis spectrophotometer (Thermo Fischer Scientific), to  $OD_{600\text{ nm}} \sim 0.323$ .

### **Statistical Analyses**

Data were analyzed using GraphPad Prism 5 (GraphPad Software, San Diego, CA). Unpaired, two-tailed Student's t-test was used to determine statistical significance unless otherwise noted.

Bacteria	Genotype	Description
<i>B. subtilis</i> DS991	<i>sinR::kan tasA::Tn10spec</i>	Overproducer/secretor of EPS
<i>B. subtilis</i> DS5187	<i>sinR::kan tasA::Tn10spec epsH::tet</i>	DS991 derivative that cannot make EPS
<i>B. subtilis</i> DK4623	<i>sinR::kan tasA::spec sdpABC::mls skf::tet lytC::cat ΔPBSX ΔSPB ΔpBS32</i>	Producer of EPS with lytic genes removed
<i>B. subtilis</i> DK6251	<i>sinR::cat tasA::cat ΔpgsB</i> Physpank-eps	Overproduce/secrete EPS under IPTG-inducible condition, γ-PGA deficient
<i>S. aureus</i> LAC	Wild-type	USA300 epidemic strain
<i>S. aureus</i> AH1263	LAC without pUSA03	Cured of Erm resistance
<i>S. aureus</i> JE2	Wild-type	Base strain for Nebraska Transposon Mutant Library, derived from LAC
<i>S. aureus</i> NE1255	<i>sek::erm</i>	Tn library mutant, Deficient for SEL-K
<i>S. aureus</i> NE1605	<i>seq::erm</i>	Tn library mutant, Deficient for SEL-Q
<i>S. aureus</i> NE1809	<i>sex::erm</i>	Tn library mutant, Deficient for SEL-X
<i>S. aureus</i> NE309	<i>USA300_1559::erm</i>	Tn library mutant, Deficient for putative type A enterotoxin

**Table 6: Bacterial Strains Used in This Study.** All *B. subtilis* strains were generously provided by Dr. Daniel B. Kearns of Indiana University. *S. aureus* LAC was from Dr. Victor J. Torres at New York University. AH1263 is from Boles and colleagues, *PLoS ONE*, 2010. All the Tn library strains are from Fey and colleagues, *mBio*, 2013.

### **Murine Model of Bloodstream Infection by *S. aureus***

Four to eight week-old C57BL/6J mice were treated with 3 to 6 mg/kg EPS in PBS by i.p. injection one day prior to and one day after systemic infection with *S. aureus*. For infection, mice were first anesthetized by i.p. injection with 250 mg/kg Avertin (MilliporeSigma), then injected with  $10^7$  CFU *S. aureus* in 100  $\mu$ L PBS through the retro-orbital venous plexus. Mice were monitored for signs of disease daily. For survival analysis, any mice that lost more than 20% body weight were considered dead to avoid utilizing any subjective criteria for assessing mortality. To assess bacterial burden, tissues were harvested from euthanized mice into 5 mL PBS and homogenized using a Polytron Brinkmann PT 10/35 homogenizer (Brinkmann Instruments, Westbury, NY). The homogenates were plated on tryptic soy agar (TSA, 1.5% agar) plates for 12 hr at 37°C to enumerate bacteria. Cleared tissue homogenate supernatants were collected by centrifugation at 3,000 x g to assess levels of local cytokine and chemokine levels. Blood samples were collected from submandibular facial veins to determine bacterial burden or to determine serum levels of cytokines and chemokines.

### **Cytokine and Chemokine Measurements**

Levels of cytokines and chemokines were determined using the cytometric bead array (CBA) flex set for IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-17A, keratinocyte-derived chemokine/C-X-C motif chemokine ligand 1 (KC/CXCL1), monocyte chemoattractant protein 1/C-C motif chemokine ligand 2 (MCP-1/CCL2), M $\Phi$  inflammatory protein 1 alpha (MIP-1 $\alpha$ /CCL3), MIP-1 $\beta$ /CCL4, regulated on activation, normal T cell expressed and secreted (RANTES/CCL5), granulocyte-M $\Phi$  colony stimulating factor (GM-CSF), granulocyte CSF (G-CSF), IFN- $\gamma$ , and TNF- $\alpha$  (BD Biosciences), according to manufacturer's specifications. Samples were analyzed using

LSRFortessa or FACSCantoII flow cytometers (BD Biosciences) and data were analyzed using FCAP array v3.0 or FlowJo V10 (BD Biosciences).

### ***S. aureus* Growth Assay**

Starter cultures of *S. aureus* were subcultured 1:100 into 200  $\mu$ L TSB containing EPS at various concentrations in a Falcon 96-well flat bottom tissue culture plate (Corning, Corning, NY). The plate was incubated at 37°C on a 250 round per minute (rpm) shaker. Growth was monitored at various time points by measuring OD<sub>550 nm</sub> using a BioTek ELx800 absorbance microplate reader (BioTek, Winooski, VT).

### ***S. aureus* Uptake and Growth Restriction by Immune Cells**

Starter culture of *S. aureus* was washed three times in 10 mL PBS then normalized to 10<sup>8</sup> CFU/mL by OD<sub>600 nm</sub> ~ 0.323. *S. aureus* inoculum was serum-opsonized for 30 min using 10% fresh mouse serum at 37°C. Four- to eight-week-old mice were treated with 3 to 6 mg/kg EPS by i.p. injection and peritoneal cells were harvested by lavage with 300 mL/kg RPMI medium. For M $\Phi$  depletion studies, 200  $\mu$ L clodronate- or PBS-loaded liposomes (VU Medisch Centrum, Amsterdam, Netherlands) were administered to ND4 Swiss Webster mice by i.p. injection 1 day prior to EPS administration. F4/80<sup>+</sup> and F4/80<sup>-</sup> peritoneal cells were purified from mice 3 days after EPS injection using the BD IMag cell separation system (BD Biosciences) according to manufacturer's specifications after incubation with anti-CD16/32 (93, BD Biosciences) and biotinylated anti-F4/80 (BM8) antibodies for 15 min on ice. F4/80<sup>+</sup> macrophages or F4/80<sup>-</sup> cells (5 x 10<sup>5</sup>), or total peritoneal cells (10<sup>6</sup>) were infected with opsonized *S. aureus* at MOI 1 in antibiotic free RPMI medium and incubated in rotating microfuge tubes at 37°C for 30 min. To enumerate internalized *S. aureus*, cells were washed three times with 1 mL PBS then lysed with 0.1% saponin (MilliporeSigma) and plated on TSA

plates for 12 hr at 37°C. Restriction of *S. aureus* growth was assessed by culturing the washed cells for 2 to 6 hr followed by quantifying *S. aureus* CFU after lysis with 0.1% saponin and plating on solid medium. For ROS experiments, 2  $\mu$ M diphenyleneiodonium (DPI, MilliporeSigma) was included in medium during the assay. To assess peritoneal cell composition, cells were first incubated with anti-CD16/32 (93) antibody for 15 min then stained with anti-CD11b-allopyocyanin (APC, M1/70), anti-F4/80-APC/cyanine 7 (APC/Cy7, BM8), anti-Ly6C-pycoerythrin (PE, HK1.4), anti-Ly6G-FITC (1A8) antibodies for flow cytometric analysis using FACSCanto II. Data were analyzed using FlowJo V10.

### **Measurement of Reactive Oxygen Species Levels**

Total peritoneal cells from PBS- or EPS-treated mice were harvested by lavage with 300 mL/kg RPMI medium 3 days after i.p. EPS injection. Cells ( $10^6$ ) were infected with opsonized *S. aureus* at MOI 1 for 30 min at 37°C in rotating microfuge tubes in the presence of 1.25  $\mu$ M CellROX Green (Thermo Fischer Scientific). For mitochondrial ROS (mtROS) measurements, 2.5  $\mu$ M MitoSOX Red (Thermo Fischer Scientific) was used instead. Cells were washed three times in 1 mL PBS then incubated with anti-CD16/32 (93) antibody for 15 min. Cells were subsequently stained with anti-CD11b-APC (M1/70) and anti-F4/80-APC/Cy7 (BM8) for flow cytometric analysis using LSRFortessa (CellROX Green) or FACSCanto II (MitoSOX Red). Data were analyzed using FlowJo V10, and median fluorescence intensity (MFI) of ROS indicators were determined after gating on CD11b<sup>high</sup>F4/80<sup>high</sup> LPMs.

### **Preparation of *S. aureus* Culture Supernatants**

Overnight starter cultures of *S. aureus* AH1263, JE2, *sek::erm*, *seq::erm*, *sex::erm*, *USA300\_1559::erm* were prepared in 5 mL antibiotic-free RPMI medium.



Starter cultures were diluted 1:100 into antibiotic-free RPMI medium and cultured for 9 hr at 37°C on a 250 rpm shaker. Supernatants were harvested by centrifugation at 3,000 x g and sterilized by passing it through a 0.2 µm filter.

### **Measurement of Reactive Nitrogen Species by Griess Test**

Peritoneal F4/80<sup>+</sup> cells from PBS- and EPS-treated mice were harvested and purified using BD IMag cell separation system 3 days after i.p. EPS injection. Cells (10<sup>5</sup>) were seeded into Falcon 96-well flat-bottom tissue culture plate in 200 µL RPMI medium per well and stimulated with 10% *S. aureus* AH1263 culture supernatant for 24 hr. Supernatant (50 µL) from the stimulated cultures were mixed with equal volume 1 % sulfanilamide (Thermo Fischer Scientific) and equal volume 0.1 % naphthylethylenediamine dihydrochloride (MilliporeSigma) and OD<sub>550nm</sub> were measured using a BioTek ELx800 absorbance microplate reader (BioTek, Winooski, VT). RNS levels were estimated using a sodium nitrite standard.

### **Superantigen-Induced Splenic T Cell Proliferation**

Total splenocytes from naïve, PBS-treated, or EPS-treated mice were labeled with 5 µM CellTrace Violet (CTV, Thermo Fischer Scientific) according to manufacturer's specifications. CTV-labeled cells (3.5 x 10<sup>5</sup>) were seeded into a Falcon 96-well flat-bottom tissue culture plate in 200 µL RPMI medium per well. Cells were stimulated with 33% *S. aureus* culture supernatants for 4 days. For peritoneal MΦ co-cultures, F4/80<sup>+</sup> cells (5 x 10<sup>4</sup>) from PBS- or EPS-treated mice, purified using the BD IMag cell separation system, were added to the wells. To assess for T cell proliferation and activation, cells were stained with anti-CD4-APC (GK1.5), anti-CD8a-PE/Cy7 (53-6.7), anti-CD25-APC/Cy7 (PC61), and anti-CD44-PE (IM7) and analyzed using LSRT Fortessa or FACSCanto II. Data were analyzed using FlowJo V10.

### **Analysis of Peritoneal Macrophage Phenotype by Flow Cytometry**

Peritoneal cells were harvested from PBS- and EPS-treated mice by lavage with 300 mL/kg RPMI medium 3 days after i.p. EPS injection. Cells were incubated with anti-CD16/32 antibody for 15 min on ice and stained with anti-CD11b-APC (M1/70), anti-F4/80-APC/Cy7 (BM8), and anti-CD206-Brilliant Violet 421 (BV421, Co68C2). Cells were fixed and permeabilized using BD Cytofix/Cytoperm solution (BD Biosciences) and stained with anti-iNOS-PE/eFluor 610 (CNXFT, Thermo Fischer Scientific) and anti-Arg-1-FITC (polyclonal Sheep IgG, Bio-Techne, Minneapolis, MN). Cells were analyzed using a LSRFortessa and the data were analyzed using FlowJo V10 by first gating on CD11b<sup>high</sup>F4/80<sup>high</sup> cells.

### **Stimulation of Splenocytes with Heat-Killed *S. aureus***

Starter cultures of *S. aureus* strains were washed three times in 10 mL PBS then normalized to 10<sup>9</sup> CFU/mL by OD<sub>600 nm</sub> ~ 1.200. *S. aureus* was then diluted in PBS to 3.5 x 10<sup>8</sup> CFU/mL and heat-killed at 65°C for 1 hr. Splenocytes from naïve WT or MyD88 KO mice were harvested and seeded (3.5 x 10<sup>5</sup> cells per well) into a Falcon 96-well flat-bottom tissue culture plate in 200 µL RPMI medium. For cell depletion experiments, target cells were incubated with anti-CD16/32 antibody for 15 min on ice, then stained with biotinylated anti-CD3ε (145-2C11), biotinylated anti-NK1.1 (PKC136), or biotinylated anti-CD11b (M1/70) antibodies prior to cell separation using the BD IMag cell separation system. Cells were stimulated by adding 10 µL heat-killed *S. aureus* preparation to MOI 10 for 24 hr. For SAg-based stimulation, cells were stimulated with 33% *S. aureus* culture supernatant for 24 or 96 hr. For neutralization experiments, 1 µg/mL anti-IL-12p40 (17.8), 10 µg/mL anti-IL-18 (YIGIF74-1G7, Bio X Cell, West Lebanon, NH), or 10 µg/mL Rat IgG<sub>2a</sub> κ isotype control (RTK2758) antibodies were

included in the medium during stimulation. Culture supernatants were collected and assessed for cytokines and chemokine levels by CBA using LSRFortessa.

### **EPS Stimulation of Peritoneal Macrophages *In Vitro***

Peritoneal cells from naïve BALB/cJ mice were harvested by lavage with 300 mL/kg RPMI medium. Cells were incubated with anti-CD16/32 (93) antibody for 15 min on ice, then stained with biotinylated anti-F4/80 (BM8) antibody prior to purification using BD IMag cell separation system. F4/80<sup>+</sup> cells ( $5 \times 10^5$ ) were seeded into a Falcon 96-well flat-bottom tissue culture plate in 200  $\mu$ L RPMI medium and stimulated with 1 or 5  $\mu$ g/mL EPS or 10 ng/mL *S. enterica* LPS (MilliporeSigma). At 24 hr, supernatants were assessed for levels of cytokines and chemokines by CBA using LSRFortessa.

### **Measurement of NF- $\kappa$ B Activation**

RAW 264.7 NF- $\kappa$ B/AP-1-secreted embryonic alkaline phosphatase reporter cell line (RAW-SEAP) was obtained from Dr. Jay Radke at Idaho Veterans Research and Education Foundation in Boise, ID. RAW-SEAP cells ( $10^5$ ) were seeded into Falcon 24-well tissue culture plate (Corning) in 500  $\mu$ L RPMI medium 1 day prior to stimulation with 0.1-1  $\mu$ g/mL EPS, 0.5-5  $\mu$ g/mL  $\Delta$ EPS, or 10 ng/mL *S. enterica* LPS. At 24 hr, culture supernatants were collected, and the endogenous alkaline phosphatase activity was heat-inactivated at 68°C for 30 min. The SEAP activity in supernatants were measured by mixing the supernatant with equal volume (50  $\mu$ L each) 1-step para-nitrophenyl phosphate (PNPP) substrate solution (Thermo Fischer Scientific) and measuring absorbance at 405 nm using a BioTek ELx800 absorbance microplate reader.

### **Analysis of IL-4R $\alpha$ Expression by Peritoneal Macrophages**

Total peritoneal cells ( $3 \times 10^5$ ) from C57BL/6J mice were seeded into Falcon 48-well tissue culture plate (Corning) in 400  $\mu$ L modified RPMI and stimulated with 5

μg/mL EPS or ΔEPS in the presence or absence of 5 μg/mL neutralizing anti-IL-10 (JES5-16E3) antibody or Rat IgG<sub>1</sub> κ isotype (RTK2071) antibody. Cells were scraped 24 hr later and incubated with anti-CD16/32 (93) antibody for 15 min on ice. Cells were then stained with anti-CD11b-APC (M1/70), anti-F4/80-APC/Cy7 (BM8), and anti-IL-4Rα-PE (Io15F8) antibodies and analyzed by flow cytometry using FACSCantoII or LSRFortessa. Data were analyzed using FlowJo V10.

### ***In Vivo* Cytokine Neutralization**

For IL-10 and TNF-α neutralization experiments, mice were treated with 250 μg neutralizing anti-IL-10 (JES5-2A5, Bio X Cell) or 500 μg neutralizing anti-TNF-α (XT3.11, Bio X Cell) antibodies by i.p. injection 1 day prior to EPS i.p. injection.

### **Flow Cytometric Characterization of Splenic Myeloid Compartment**

WT or TLR4 KO mice were treated with PBS or EPS by i.p. injection. Splenocytes were harvested the next day and incubated with anti-CD16/32 (93) antibody for 15 min on ice. Cells were stained with anti-CD11b-APC/Cy7 (M1/70), anti-CD11c-APC (N418), anti-Ly6C-PE (HK1.4), and anti-Ly6G-FITC (1A8). Cells were analyzed using LSRFortessa. Data were analyzed using FlowJo V10.

### **Stimulation of Bone Marrow Neutrophils**

Mouse tibias and femurs were flushed with 3 mL RPMI medium to harvest bone marrow cells. Bone marrow cells were layered over a Histopaque gradient established using Histopaque 1077 layered over Histopaque 1119 (MilliporeSigma). Neutrophils were isolated by centrifugation at 800 x g for 30 min at room temperature without brakes. The interface layer containing bone marrow neutrophils (BM-PMNs) were carefully collected and washed twice in 10 mL modified RPMI medium. BM-PMNs (10<sup>5</sup>) were seeded into Falcon 96-well flat-bottom plate in 200 μL modified RPMI and

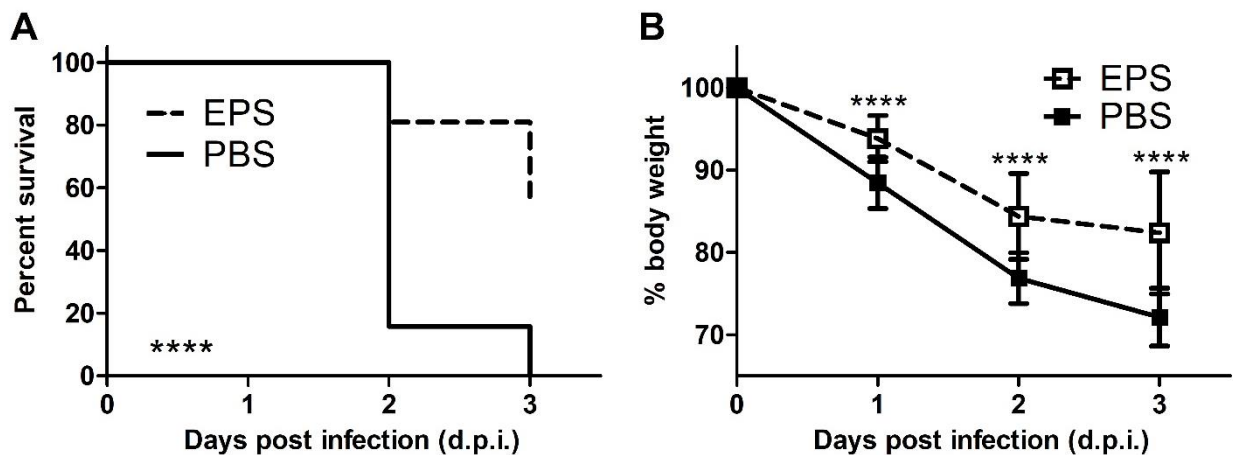
stimulated with 5 µg/mL EPS or ΔEPS, or 10 ng/mL *S. enterica* LPS for 24 hr. Culture supernatants were collected and assessed for cytokine and chemokine levels using CBA.

## CHAPTER THREE

### RESULTS

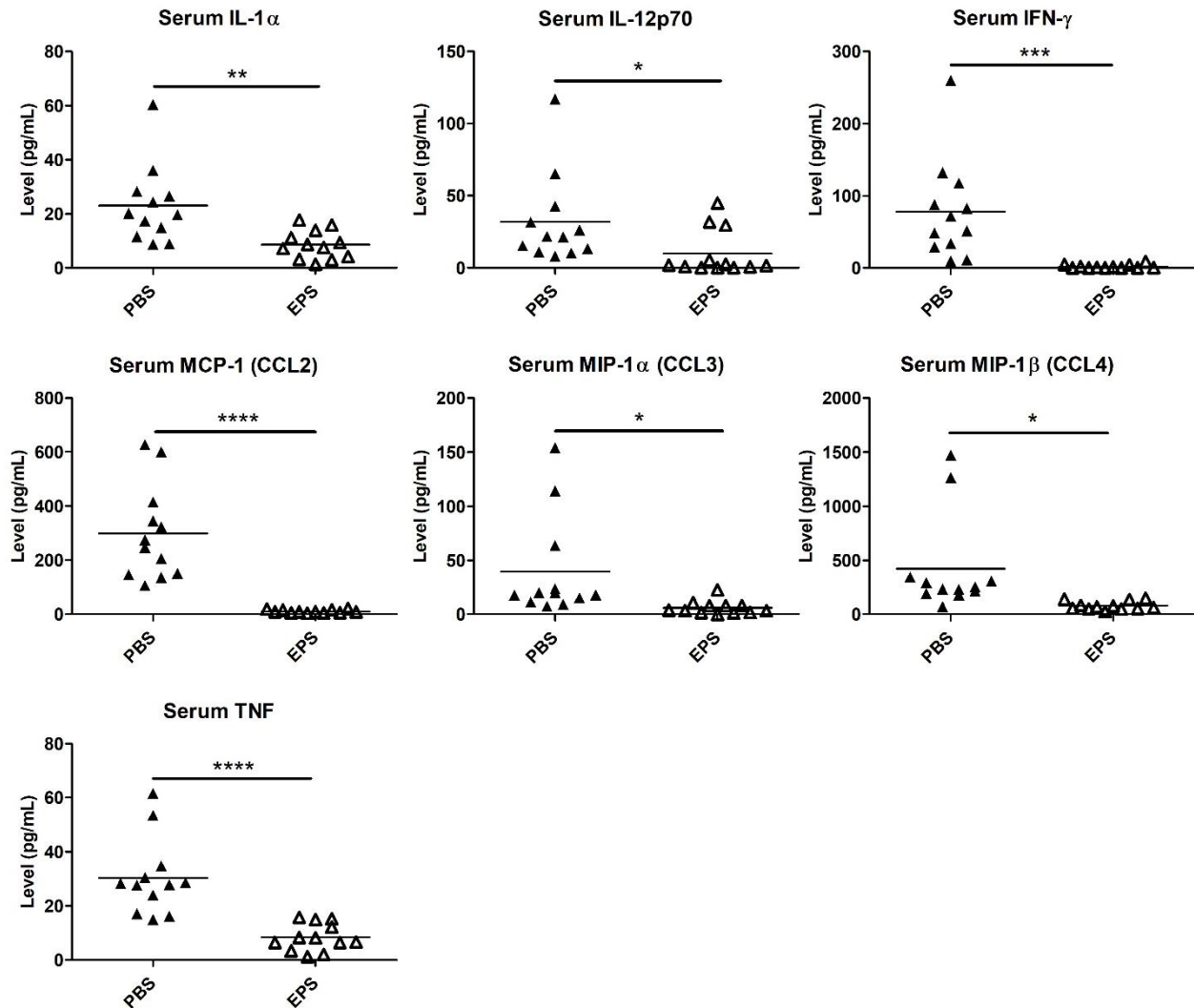
#### ***B. subtilis*-derived EPS Attenuates Bloodstream Infection by *S. aureus***

To test our hypothesis that EPS could limit inflammation during systemic infections to improve outcomes, we administered EPS to mice one day prior to, and one day following infection with *S. aureus* USA300 LAC, a clone of an epidemic community-acquired (CA)-MRSA strain from Los Angeles County (Miller et al., 2005), through the retro-orbital venous plexus. This mimics direct seeding of the bloodstream as it could occur during invasive surgical procedures or intravenous (IV) drug use. We found that whereas all PBS-treated mice died by 3 d.p.i., more than 50% of EPS-treated mice survived (**Figure 3A**).



**Figure 3: Effect of EPS on *S. aureus* Bloodstream Infection Outcomes in Mice.** Mice were treated with EPS on -1 and 1 d.p.i. and infected systemically with *S. aureus*. **A.** Survival analysis. Any mice with more than 20% body weight loss were considered dead. Data analyzed using Mantel-Cox test. **B.** % body weight. Error bars represent standard deviation (SD). N = 19 (PBS), 21 (EPS). Data pooled from 6 independent experiments. \*\*\*\*P<0.0001. Adopted from Paik and colleagues, *Infection and Immunity*, 2019.

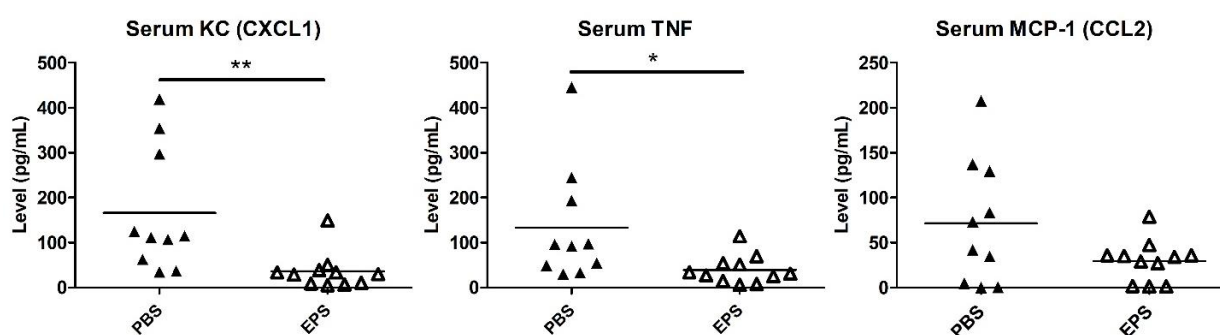
We also found that EPS-treated mice lost less weight compared to PBS-treated mice (**Figure 3B**), suggesting that the overall burden of disease was reduced.



**Figure 4: Effect of EPS on Systemic Inflammation During *S. aureus* Bloodstream Infection at 1 d.p.i.** Serum levels of cytokines and chemokines were measured in PBS- or EPS-treated mice after systemic *S. aureus* infection on 1 d.p.i. using CBA. Each triangle represents one mouse. Bars represent mean. N = 12. Data pooled from 3 independent experiments. \* $P < 0.05$ . \*\* $P < 0.01$ . \*\*\* $P < 0.001$ . \*\*\*\* $P < 0.0001$ . Adopted from Paik and colleagues, *Infection and Immunity*, 2019.

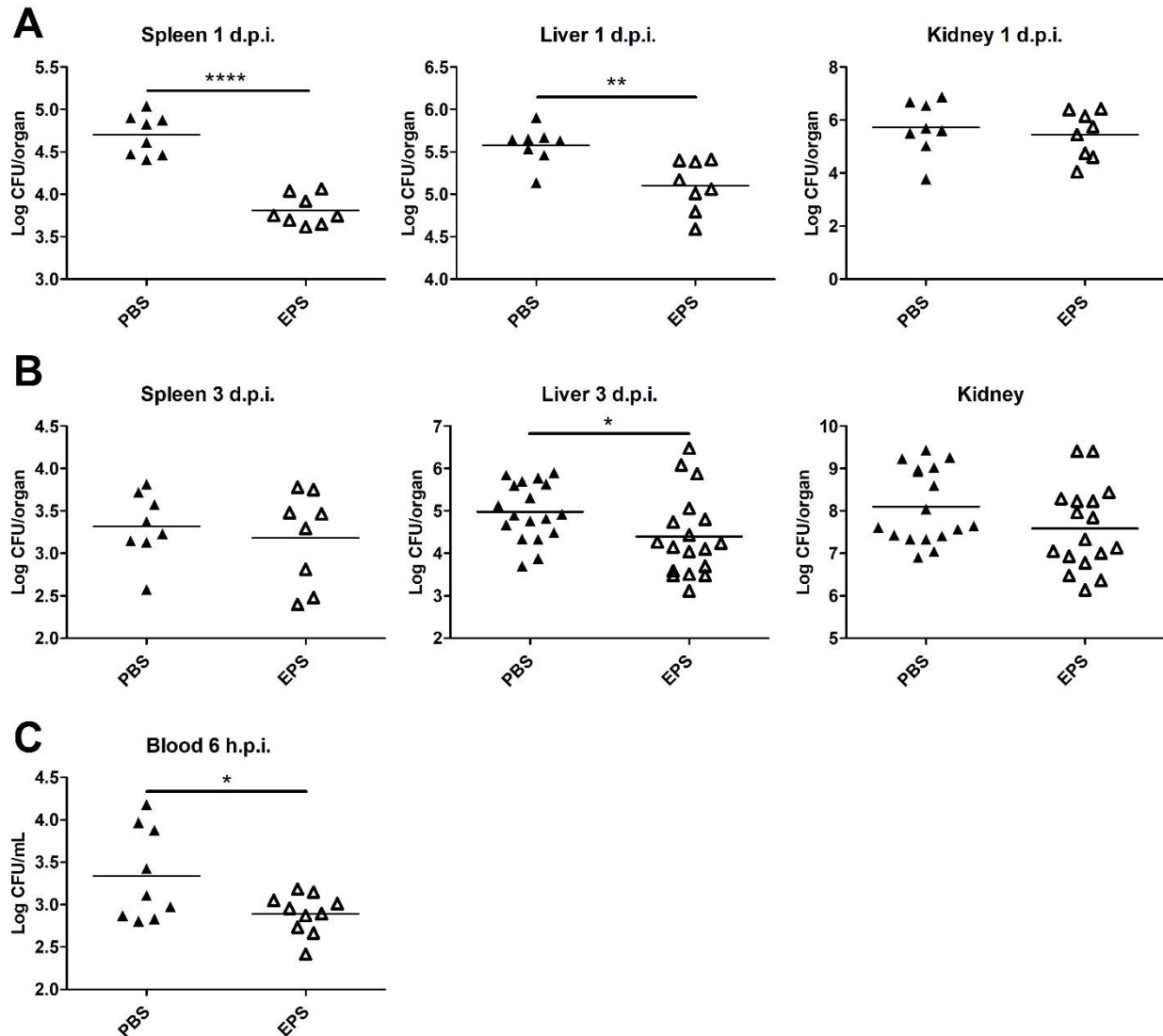
We could notice the beneficial effect of EPS even with a single dose early during disease, since even at 1 d.p.i., before the second dose of EPS was administered, EPS-treated mice had lost less weight compared to PBS-treated mice (**Figure 3B**). This suggests that pre-treatment with EPS reduces the burden of disease induced by systemic *S. aureus* infection, increasing host survival.

Given that EPS has anti-inflammatory properties (Paynich et al., 2017), we assessed the levels of serum cytokines and chemokines in EPS-treated mice. Indeed, serum levels of IL-1 $\alpha$ , IL-12p70, IFN- $\gamma$ , MCP-1, MIP-1 $\alpha$ , MIP-1 $\beta$ , and TNF were reduced in EPS-treated mice compared to PBS-treated mice at 1 d.p.i. (**Figure 4**), indicating that EPS limits systemic inflammation early during disease. We did not observe any differences in serum IL-6 and KC levels, and we could not detect IL-2 at this time (**data not shown**). At 3 d.p.i., time-point representing peak disease, the serum levels of KC and TNF were reduced while serum levels of MCP-1 trended lower as well (**Figure 5**). No differences in serum levels of IL-6, IL-12p70, MIP-1 $\alpha$ , and MIP-1 $\beta$  could be observed, and IFN- $\gamma$  was not detectable at this time (**data not shown**). This suggests that a single dose of EPS reduces systemic inflammation during systemic *S. aureus* infection.



**Figure 5: Effect of EPS on Systemic Inflammation During *S. aureus* Bloodstream Infection at 3 d.p.i.** Serum levels of cytokines and chemokines were measured in PBS- and EPS-treated mice after systemic *S. aureus* infection on 3 d.p.i. using CBA. Each dot represents one mouse. Bars represent mean. N = 10 (PBS), 11 (EPS). Data pooled from 3 independent experiments. \*P<0.05. \*\*P<0.01. Adopted from Paik and colleagues, *Infection and Immunity*, 2019.





**Figure 6: Effect of EPS on *S. aureus* Burden in Organs During Systemic Infection.** *S. aureus* burden in organs were assessed by determining CFU *S. aureus* in organ homogenates by dilution plating, from PBS- and EPS-treated mice following systemic infection. **A.** CFU *S. aureus* at 1 d.p.i. Data pooled from 2 independent experiments. N = 8. **B.** CFU *S. aureus* at 3 d.p.i. Data pooled from 2 (spleen) or 6 (liver and kidney) independent experiments. N = 8 (spleen), 18 (liver), 17 (kidney). **C.** CFU *S. aureus* in blood at 6 h.p.i. Each triangle represents one mouse. Bars represent mean. Data pooled from 2 independent experiments. N = 9 (PBS), 10 (EPS). \* $P < 0.05$ . \*\* $P < 0.01$ . \*\*\*\* $P < 0.0001$ . Adopted from Paik and colleagues, *Infection and Immunity*, 2019.

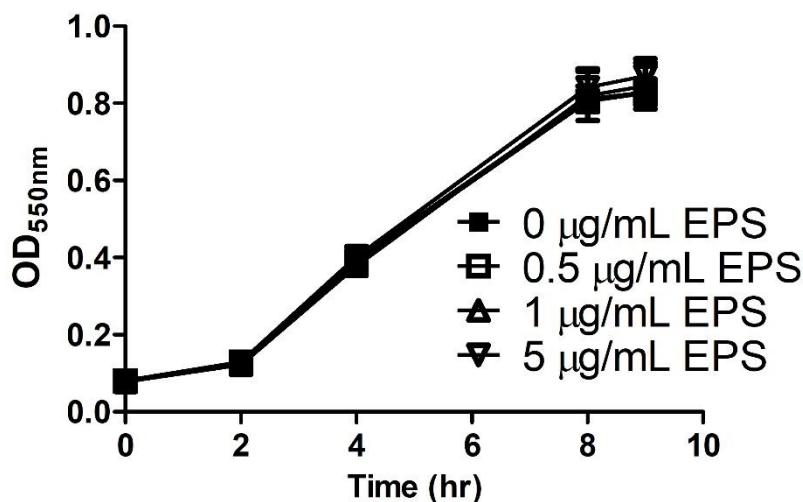
While EPS did not alter pathogen colonization in the *C. rodentium*-induced colitis

model (Jones et al., 2014), we still tested if EPS alters *S. aureus* colonization during infection by assessing bacterial load within organs. To our surprise, EPS-treated mice had 8-fold reduced CFU *S. aureus* in the spleen and 3-fold reduced CFU *S. aureus* in the liver at 1 d.p.i. (**Figure 6A**), suggesting that EPS reduces bacterial burden. A 3-fold decrease in CFU *S. aureus* was noted in the liver at 3 d.p.i., although not statistically significant (**Figure 6B**). No difference in the bacterial load was observed in the spleen at 3 d.p.i., and in the kidney at all time points assessed (**Figure 6A and B**). Since the beneficial effect of EPS was noted as early as 1 d.p.i., we tested if EPS-treated mice have reduced bacterial burden at an earlier time point by assessing the circulating numbers of *S. aureus*. We found that EPS-treated mice had 3-fold lower CFU *S. aureus* in the blood as early as 6 hours post infection (h.p.i.) (**Figure 6C**). Together, these data suggest that EPS reduces bacterial burden early during systemic infection by *S. aureus*.

#### **EPS Protection from *S. aureus* Bloodstream Infection Through TLR4**

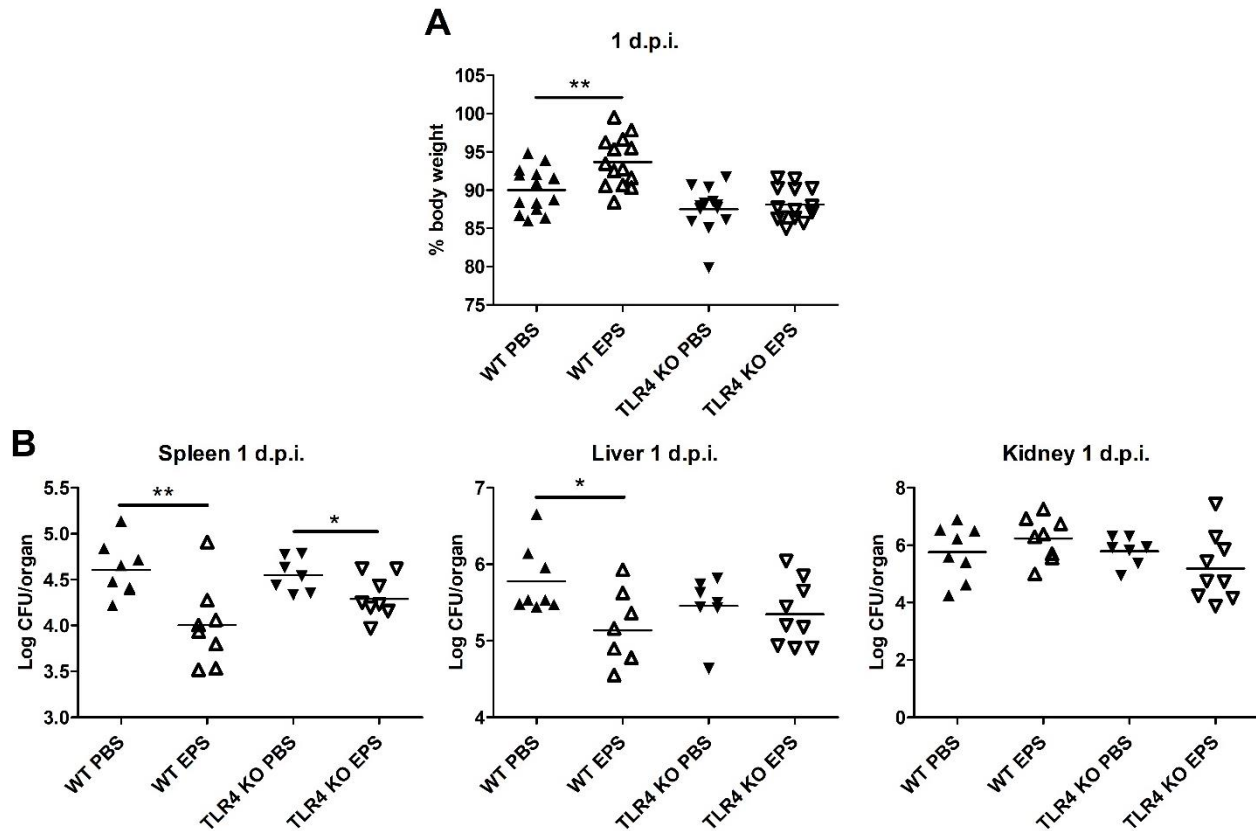
EPS protects mice from *C. rodentium*-induced colitis through the inhibition of T cell activation that drives disease, and any changes in pathogen colonization could not be observed in that model (Jones et al., 2014). In *S. aureus* bloodstream infection, EPS-treated mice had reduced bacterial burden in multiple organs (**Figure 6**), indicating that in this model, EPS had an antimicrobial effect. *B. subtilis* has been demonstrated to produce bacteriocins, including those with antimicrobial activity against Gram-positive pathogens like *L. monocytogenes* and *S. aureus* (Liu et al., 2015; Shelburne et al., 2007). Therefore, one possibility for the reduced bacterial load in EPS-treated mice is that our EPS preparations contain antimicrobial properties. We tested this by culturing *S. aureus* in the presence of EPS *in vitro* and found that EPS did not affect *S. aureus* growth

(**Figure 7**), suggesting that the EPS preparation does not have direct antimicrobial activity against *S. aureus*.



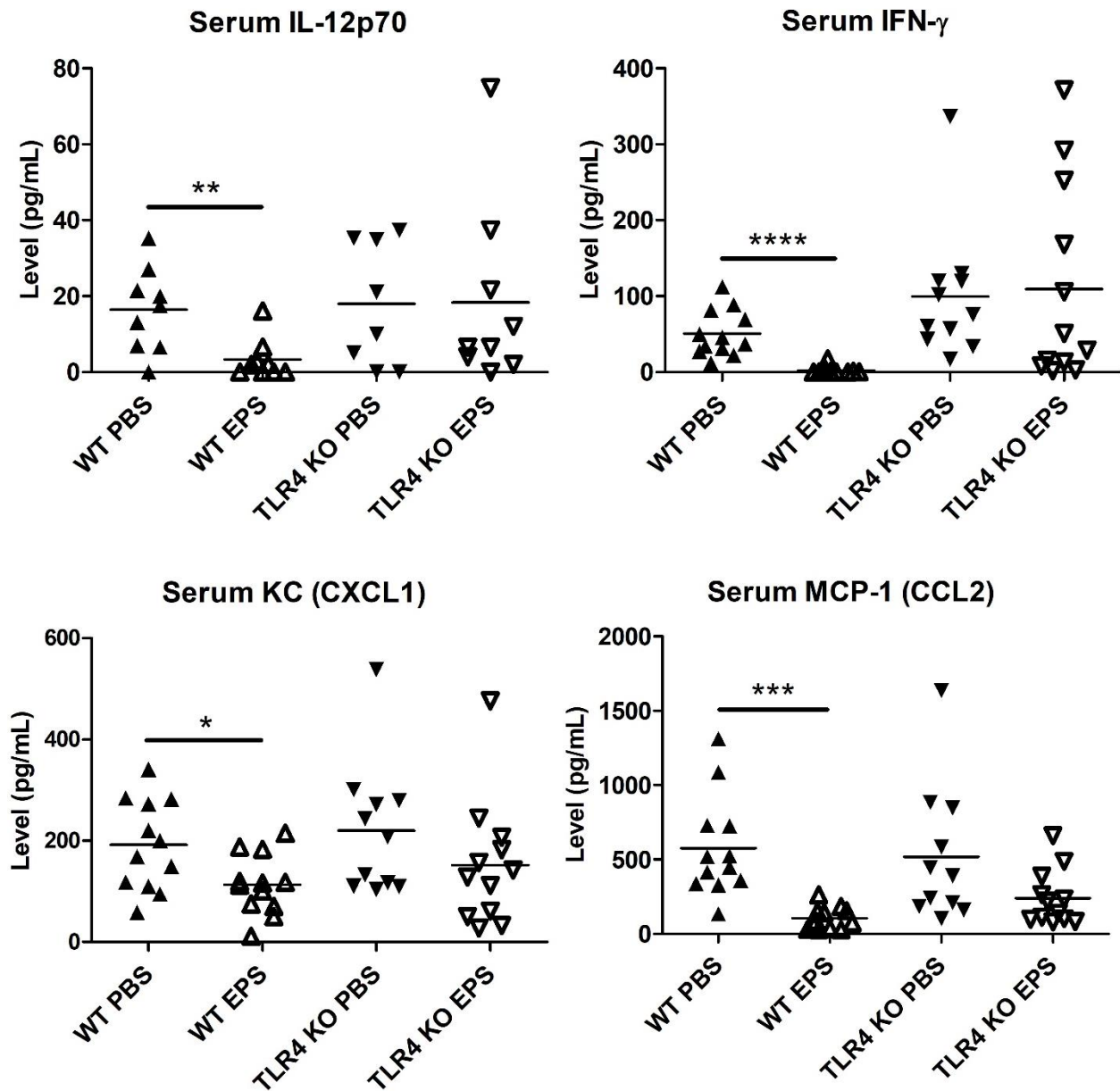
**Figure 7: Effect of EPS on *S. aureus* Growth *In Vitro*.** Overnight culture of *S. aureus* AH1263 was subcultured 1:100 in TSB containing noted concentrations of EPS and growth was monitored over time. Error bars represent SD. N = 3.

The other possible explanation for EPS reducing pathogen burden during infection is through indirect mechanisms, by modulating the host immune system, for example. The protection from *C. rodentium*-induced colitis by EPS requires TLR4 and MyD88 (Jones et al., 2014; Paynich et al., 2017), suggesting that EPS modulates host immunity through TLR4-MyD88 signaling. To test if protection from *S. aureus* bloodstream infection by EPS occurs through immune modulation, we asked whether TLR4 is required for EPS protection using TLR4-deficient mice. We treated wild-type and TLR4 KO mice with EPS one day prior to systemic infection with *S. aureus* and found that while EPS-treated wild-type mice lost less weight compared to PBS-treated mice at 1 d.p.i., this difference was not observed between PBS- and EPS-treated TLR4 KO mice (**Figure 8A**).



**Figure 8: Role of TLR4 on EPS Protection from *S. aureus* Bloodstream Infection.** Wild-type (WT) or TLR4 KO mice were treated with PBS or EPS one day before *S. aureus* bloodstream infection. **A.** % body weight at 1 d.p.i. Data pooled from 4 independent experiments. N = 13 (TLR4 KO PBS), 14 (all groups). **B.** CFU *S. aureus* in organ homogenates at 1 d.p.i. Each triangle represents data from one mouse. Bars represent mean. Data pooled from 2 independent experiments. N = 8 (WT), 7 (TLR4 KO PBS), 9 (TLR4 KO EPS). \*P<0.05. \*\*P<0.01.

We also assessed the bacterial burden within organs and found that while EPS-treated WT mice had reduced CFU *S. aureus* in spleen and liver at 1 d.p.i., EPS-treated TLR4 KO mice did not have such a reduction in splenic bacterial burden and no difference in hepatic CFU *S. aureus* compared to PBS-treated TLR4 KO mice (**Figure 8B**). As before, we did not see any difference in CFU *S. aureus* in kidney between all groups (**Figure 8B**). These data suggest that the reduction of the bacterial burden during systemic *S. aureus* infection depends on the modulation of host immunity by EPS through TLR4.



**Figure 9: Role of TLR4 on EPS Inhibition of Early Systemic Inflammation During *S. aureus* Bloodstream Infection.** WT or TLR4 KO mice were treated with EPS 1 day prior to systemic *S. aureus* bloodstream infection. Serum levels of cytokines and chemokines were determined using CBA. Each triangle represents data from one mouse. Bars represent mean. Data pooled from 3 independent experiments. N = 8 (TLR4 KO PBS, IL-12p70), 9 (all groups, IL-12p70), 11 (TLR4 KO PBS, all groups), 12 (all groups). \*P>0.05. \*\*P>0.01. \*\*\*P>0.001. \*\*\*\*P>0.0001.

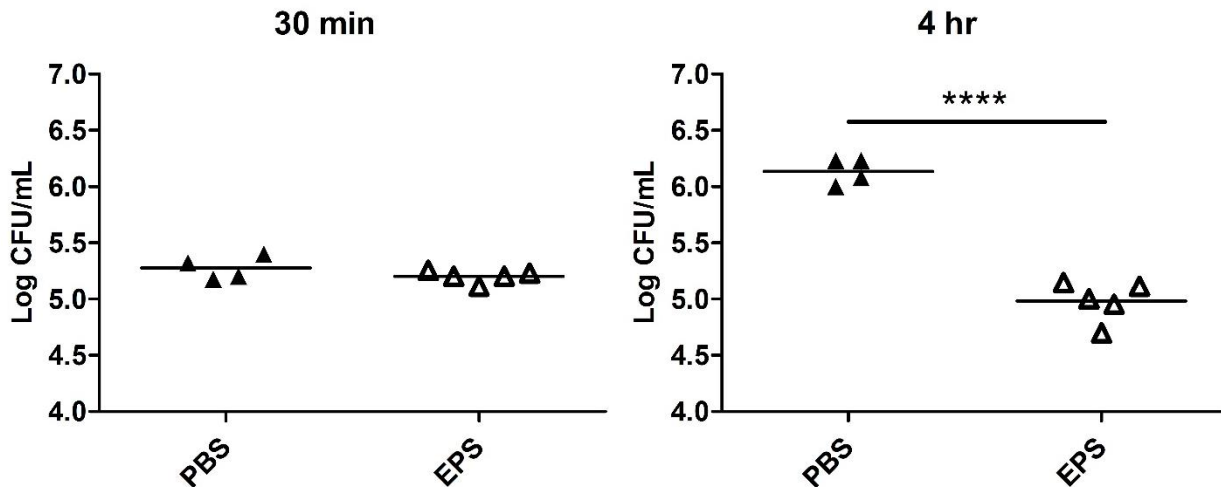
We also tested if EPS affects inflammation during disease in a TLR4-dependent manner

by measuring serum levels of cytokines and chemokines from the infected mice in

**Figure 8.** The levels of pro-inflammatory cytokines and chemokines IL-12p70, IFN- $\gamma$ , KC, and TNF were reduced in EPS-treated WT mice, but not in EPS-treated TLR4 KO mice (**Figure 9**), indicating that the reduction in systemic inflammation by EPS during systemic *S. aureus* infection requires TLR4. Together, these data indicate that EPS protects hosts from systemic *S. aureus* infection by modulating the host immune response through TLR4, resulting in both reduced bacterial burden and systemic inflammation.

### **Anti-Bacterial Activity of EPS-Induced Peritoneal Macrophages**

The initial host response to *S. aureus* infection is characterized by an influx of innate immune cells, particularly neutrophils and M $\Phi$ s, designed for phagocytosis of invading pathogens and intracellular killing. EPS protects mice from systemic *S. aureus* infection, reducing bacterial load early during disease through modulation of host immunity through TLR4. In addition, EPS is known to exert its functions through M $\Phi$ s in *C. rodentium*-induced colitis, albeit through the anti-inflammatory polarization of M $\Phi$ s (Paynich et al., 2017). Therefore, we hypothesized that EPS might reduce bacterial burden during infection by bolstering the two primary antimicrobial functions of innate immune cells: by increasing bacterial uptake or by increasing intracellular killing. We first incubated total peritoneal cells from PBS- and EPS-treated mice with opsonized *S. aureus* for 30 min to assess bacterial uptake and found no difference between the number of *S. aureus* CFU taken up by cells (**Figure 10**), indicating that EPS did not affect bacterial uptake by immune cells.



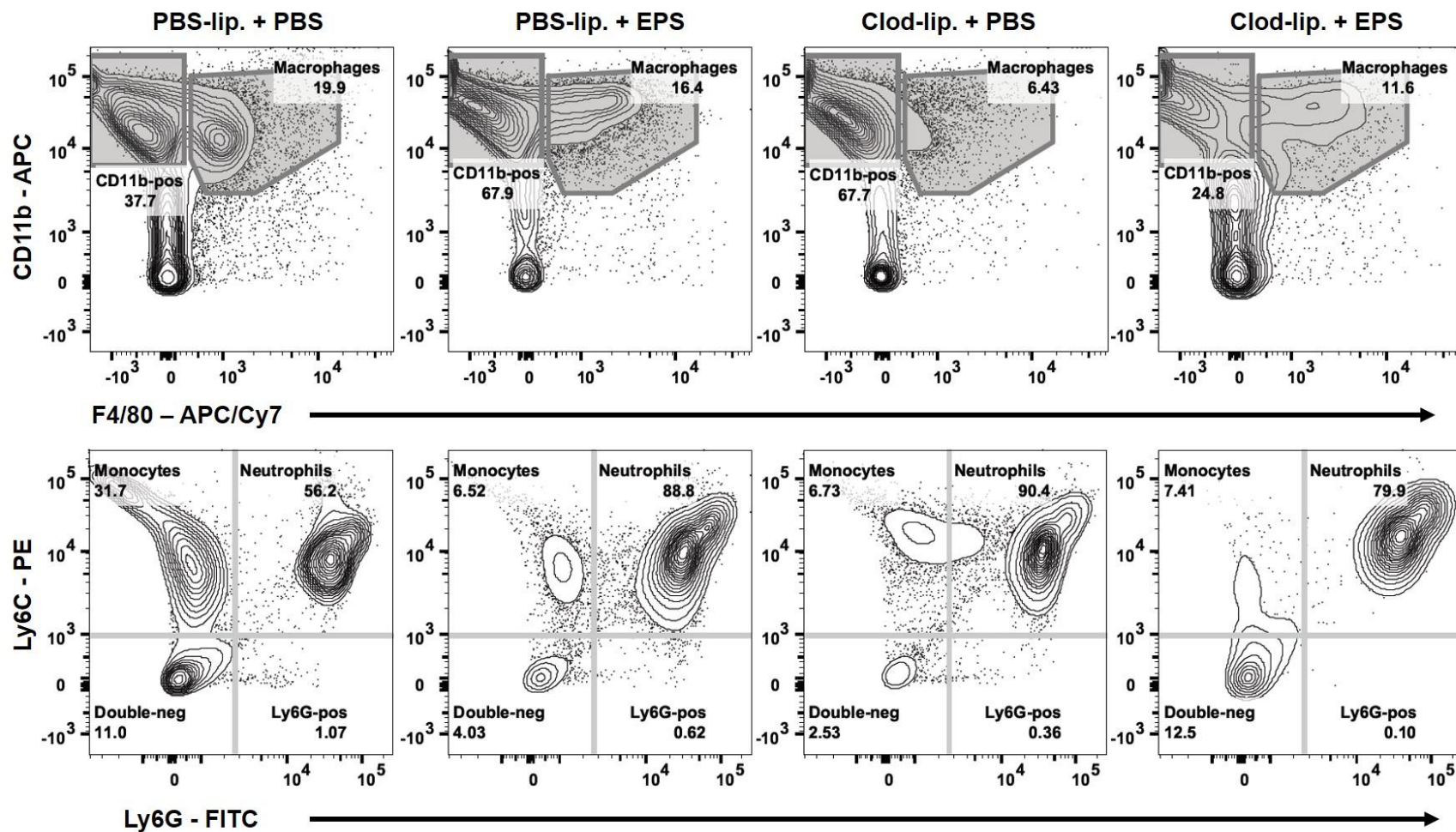
**Figure 10: Effect of EPS on Peritoneal Cell Restriction of *S. aureus* Growth.** Peritoneal cells ( $10^6$ ) from PBS- or EPS-treated Balb/cJ mice were infected *ex vivo* with opsonized *S. aureus* at MOI 1. CFU internalized *S. aureus* was assessed at 30 min after washing cells of free bacterium (left). Washed cells were further cultured and CFU *S. aureus* was assessed at 4 hr (right). Each triangle represents data from one mouse. Bar represents mean. N = 4 (PBS), 5 (EPS). \*\*\*\*P>0.0001.

To test if EPS enhances immune cell-mediated killing of internalized *S. aureus*, we continued to culture washed cells and found that by 4 hr, cells from EPS-treated mice had less CFU *S. aureus* than PBS-treated mice (**Figure 10**), suggesting that EPS enhances immune cell restriction of *S. aureus* growth.

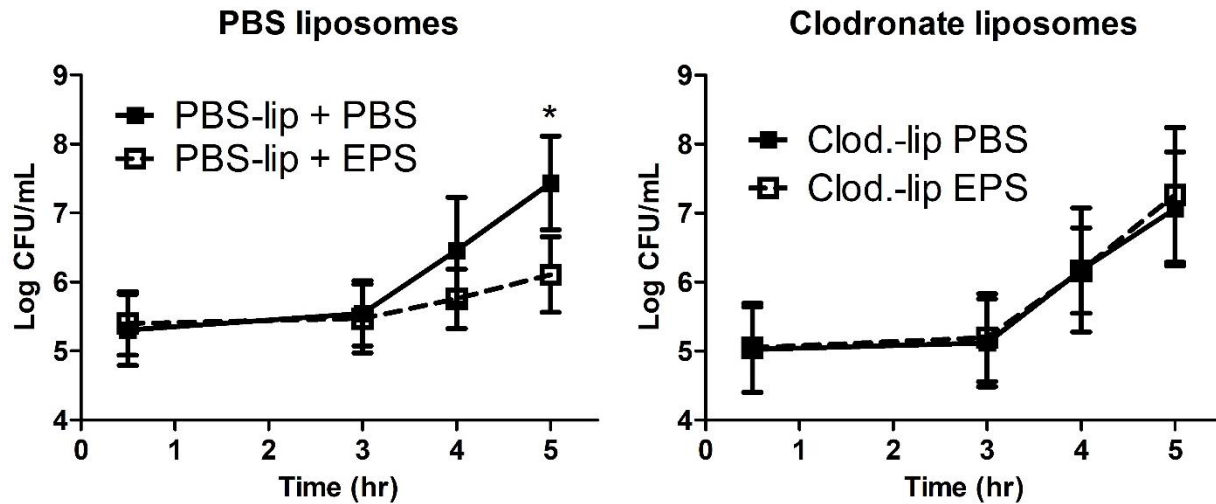
The two principal immune cells that mediate phagocytic killing of bacteria are neutrophils and MΦs. Protection from *C. rodentium*-induced colitis by EPS requires MΦs, since i.p. administration of clodronate-loaded liposomes to deplete MΦs negated the protective effects of EPS (Paynich et al., 2017). Therefore, we took the same approach to test if MΦs were responsible for the restriction of *S. aureus* growth in EPS-treated mice. We administered clodronate-loaded liposomes to mice prior to PBS or EPS treatment which resulted in a reduction in percent peritoneal CD11b<sup>+</sup>F4/80<sup>+</sup> cells (MΦs) in both PBS- and EPS-treated mice (**Figure 11**). We also observed an increase in the percent CD11b<sup>+</sup>Ly6G<sup>+</sup>F4/80<sup>-</sup> cells (PMNs) in EPS-treated mice and in both clodronate-

loaded liposome treated mice (**Figure 11**). We infected these cells with opsonized *S. aureus* to test their capacity to restrict *S. aureus* growth as before and found that while cells from mice treated with EPS and control liposomes had lower CFU *S. aureus* by 5 hr, cells from mice with EPS and clodronate-loaded liposomes did not (**Figure 12**), indicating that peritoneal MΦs are required for restriction of *S. aureus* growth *in vitro*.





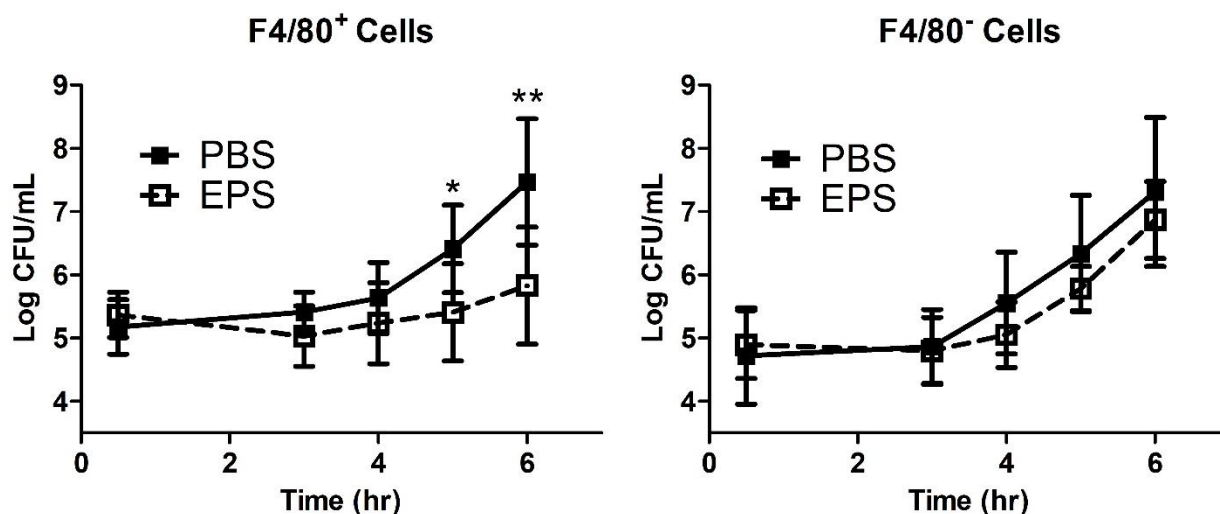
**Figure 11: *In Vivo* Depletion of Peritoneal Macrophages Using Clodronate-Loaded Liposomes.** ND4 Swiss Webster mice were given 200  $\mu$ L PBS- or clodronate-loaded liposomes by i.p. injection 1 day prior to i.p. EPS injection. Peritoneal cells were harvested 1 day later and analyzed by flow cytometry. Representative data from 2 experiments.



**Figure 12: Effect of EPS on Restriction of *S. aureus* Growth in the Absence of MΦs.** Peritoneal cells ( $10^6$ ) were harvested from EPS-treated ND4 Swiss Webster mice, depleted of MΦs by pre-treatment with clodronate-loaded liposomes 1 day before treatment with EPS, 1 day after EPS and were infected with opsonized *S. aureus* at MOI 1. After washing, growth of internalized *S. aureus* was monitored over time. Error bars represent SD. Data pooled from 4 independent experiments. N = 4. \*P<0.05.

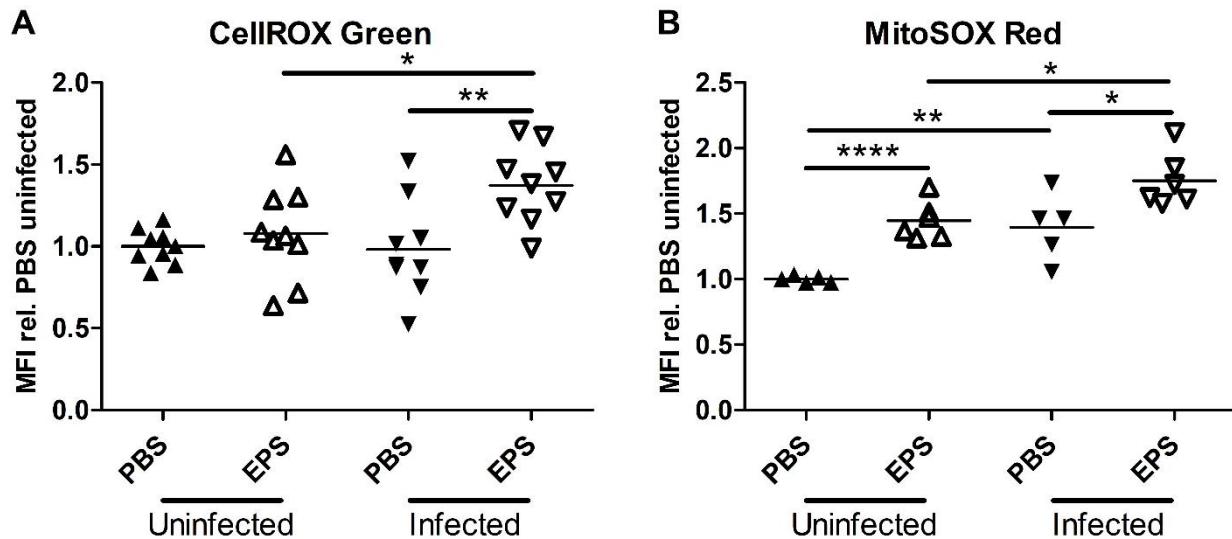
EPS is thought to polarize MΦs into the anti-inflammatory M2 phenotype, which in turn limits the activation of T cells to protect hosts from inflammatory diseases such as *C. rodentium*-induced colitis (Paynich et al., 2017). Generally, M2 MΦs are not associated with antimicrobial functions. In fact, Arg-1-expressing MΦs have been found associated with *S. aureus* persistence in a murine model of catheter-associated biofilm infection (Thurlow et al., 2011). Therefore, we hypothesized that two reasons underlie the apparent anomaly of EPS-induced MΦs restricting *S. aureus* growth *ex vivo*. For one, EPS-induced M2 MΦs are typically detectable 3 days after i.p. EPS injection (Paynich et al., 2017), so at earlier time points, the MΦs may take on pro-inflammatory characteristics like antimicrobial activity. The other is that since MΦs modulate their functions in response to environmental cues, the EPS-induced M2 MΦs may increase their antimicrobial activity in response to infection by *S. aureus*. To test this, we isolated

peritoneal F4/80<sup>+</sup> MΦs from PBS- or EPS-treated mice at 3 days after i.p. EPS injection and tested their capacity to restrict *S. aureus* growth *ex vivo*. We found that whereas peritoneal F4/80<sup>+</sup> cells from EPS-treated mice had lower CFU *S. aureus* than F4/80<sup>+</sup> cells from PBS-treated mice by 5 hr, F4/80<sup>-</sup> cells from PBS- and EPS-treated mice did not show any difference in CFU *S. aureus* (**Figure 13**), confirming that EPS-induced peritoneal MΦs, previously demonstrated to be anti-inflammatory M2 MΦs (Paynich et al., 2017), restrict *S. aureus* growth.



**Figure 13: Effect of EPS on Peritoneal MΦ-Mediated Restriction of *S. aureus* Growth.** Peritoneal F4/80<sup>+</sup> and F4/80<sup>-</sup> cells ( $5 \times 10^5$ ) harvested from PBS- or EPS-treated mice 3 days after EPS injection were infected with opsonized *S. aureus* at MOI 1. After washing, growth of internalized *S. aureus* was assessed over time. Error bars represent SD. Data pooled from 4 independent experiments. N = 7 (F4/80<sup>+</sup> PBS), 8 (F4/80<sup>+</sup> EPS), 5 (F4/80<sup>-</sup> PBS), 6 (F4/80<sup>-</sup> EPS). \*P<0.05. \*\*P<0.01. Adopted from Paik and colleagues, *Infection and Immunity*, 2019.

A classic mechanism by which phagocyte kill bacteria or restrict their growth internally is the production of ROS and RNS. After uptake, phagocytes use NADPH oxidase that catalyzes the reaction of NADPH and O<sub>2</sub> to generate O<sub>2</sub><sup>-</sup>, which is then used to generate a variety of reactive oxidants that damage microbial processes (Sbarra and Karnovsky, 1959; Segal, 1987; Xie et al., 1992).



**Figure 14: Effect of EPS on Peritoneal MΦ Production of ROS.** Peritoneal cells were harvested from PBS- or EPS-treated mice 3 days after EPS injection. Cells ( $10^6$ )s were incubated with **A.** CellROX Green or **B.** MitoSOX Red ROS indicators in the presence of absence of infection with opsonized *S. aureus* at MOI 1. MFI of ROS indicators on CD11b<sup>high</sup>F4/80<sup>high</sup> cells were assessed by flow cytometry. Each triangle represents data from one mouse. Bars represent mean. Data pooled from 5 (CellROX) or 2 (MitoSOX) independent experiments. N = 9 (CellROX), 5 (MitoSOX, PBS), 6 (MitoSOX, EPS). \*P<0.05. \*\*P<0.01. \*\*\*\*P<0.0001. Adopted from Paik and colleagues, *Infection and Immunity*, 2019.

To determine if EPS-induced MΦs use ROS to restrict the growth of internalized *S.*

*aureus*, we measured ROS levels in EPS-induced MΦs after infection with opsonized *S.*

*aureus ex vivo* using a fluorescent ROS indicator, CellROX Green. There was no

difference in CellROX Green staining between CD11b<sup>high</sup>F4/80<sup>high</sup> MΦs from PBS- and

EPS-treated mice freshly isolated, but upon infection with *S. aureus ex vivo*, MΦs from

EPS-treated mice had increased CellROX Green staining compared to MΦs from PBS-

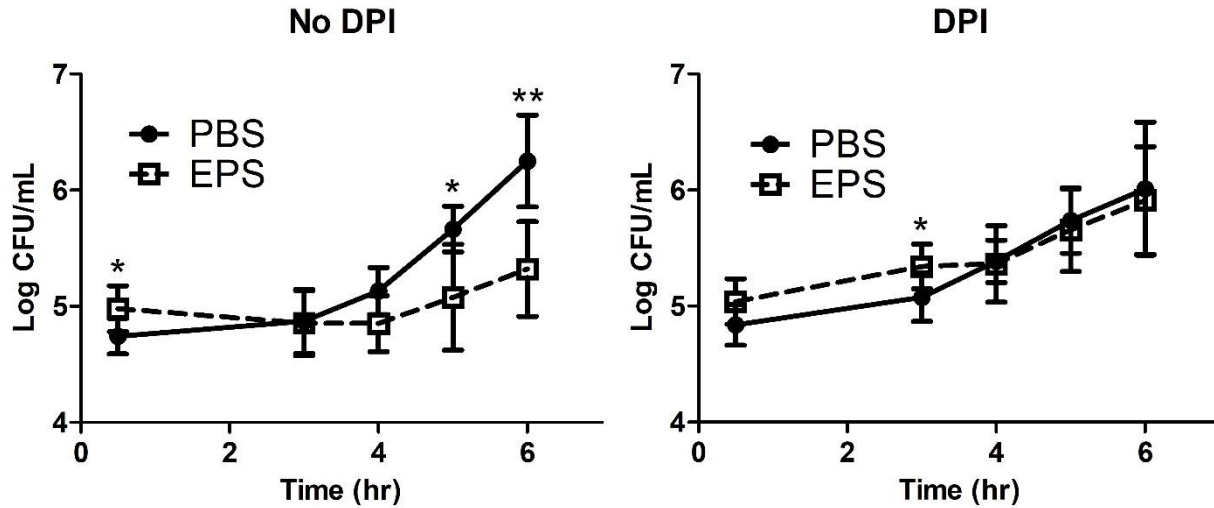
treated mice, which failed to increase CellROX staining (**Figure 14A**). This suggests

that EPS-induced MΦs, while not increased in ROS levels at baseline, can increase ROS

during infection. Another source of ROS is the mitochondria, thought to be produced

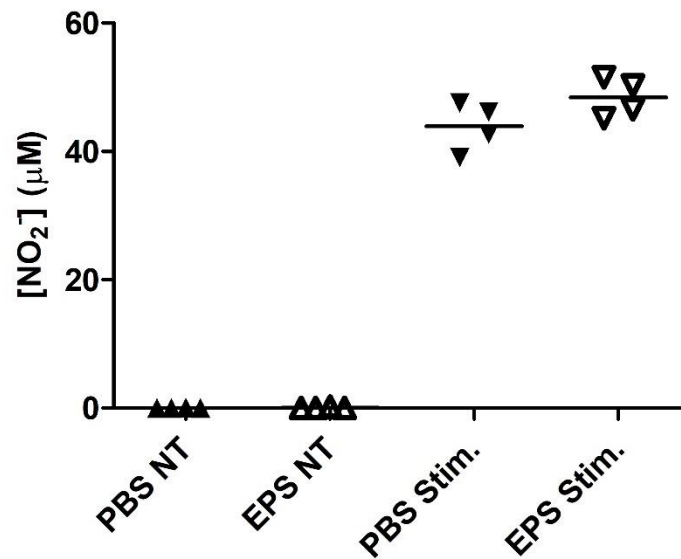
through mitochondrial respiratory metabolic processes (Boveris et al., 1976; Turrens

and Boveris, 1980).



**Figure 15: Restriction of *S. aureus* Growth by EPS-Induced Peritoneal MΦs and ROS Production.** Peritoneal F4/80<sup>+</sup> cells ( $5 \times 10^5$ ) from PBS- or EPS-treated mice were harvested 3 days after i.p. EPS. Cells were infected with opsonized *S. aureus* at MOI 1, then monitored for *S. aureus* growth after washing in the presence or absence of 2  $\mu$ M DPI in the medium. Error bars represent SD. Data pooled from 4 independent experiments. N = 6. \*P<0.05. \*\*P<0.01. Adopted from Paik and colleagues, *Infection and Immunity*, 2019.

This mtROS can be used to augment phagocyte antibacterial activity (West et al., 2011), and mtROS was recently reported to play a role in MΦ responses against *S. aureus* (Abuaita et al., 2018). M2 MΦs are associated with mitochondrial metabolic processes compared to M1 MΦs that rely more on glycolysis (Huang et al., 2014; 2016; Rodríguez-Prados et al., 2010), and tissue-derived mtROS was demonstrated to promote differentiation of M2 MΦs *in vivo* (Formentini et al., 2017). Given this, we hypothesized that EPS-induced peritoneal MΦs might harbor increased levels of mtROS. To test this, we stained the peritoneal cells as in **Figure 14A** with MitoSOX Red mtROS indicator and found that CD11b<sup>high</sup>F4/80<sup>high</sup> LPMs from EPS-treated mice had higher levels MitoSOX Red staining compared to PBS-treated mice at baseline (**Figure 14B**), indicating that EPS-induced MΦs have increased mtROS.



**Figure 16: Effect of EPS on MΦ Production of RNS.** F4/80<sup>+</sup> cells (10<sup>5</sup>) from PBS- and EPS-treated mice were stimulated with 10% *S. aureus* culture supernatant and RNS levels were determined using the Griess test. Each triangle represents data from one mouse. Bars represent mean. Data pooled from 2 independent experiments. N = 4.

Both cells had increased MitoSOX Red staining upon infection with *S. aureus* beyond baseline levels, and EPS-induced MΦs had mtROS levels that were even higher (**Figure 14B**), consistent with the previous reports that mtROS plays a role in MΦ responses to *S. aureus* infection. To confirm that this increase in ROS production was responsible for restriction of *S. aureus* growth by EPS-induced MΦs, we repeated the restriction assay as in **Figure 13** in the presence of DPI, a general ROS inhibitor (Cross and Jones, 1986). We found that in the presence of DPI, restriction of *S. aureus* growth by EPS-induced MΦs was abrogated (**Figure 15**). We also measured RNS production from EPS-induced MΦs using the Griess test. However, supernatants from the MΦ cultures from *S. aureus* infection *ex vivo* from **Figure 13** did not have detectable RNS levels (**data not shown**). We then stimulated F4/80<sup>+</sup> peritoneal cells from PBS- and EPS-treated mice with culture supernatants of *S. aureus* to compare their RNS-producing capacity and found



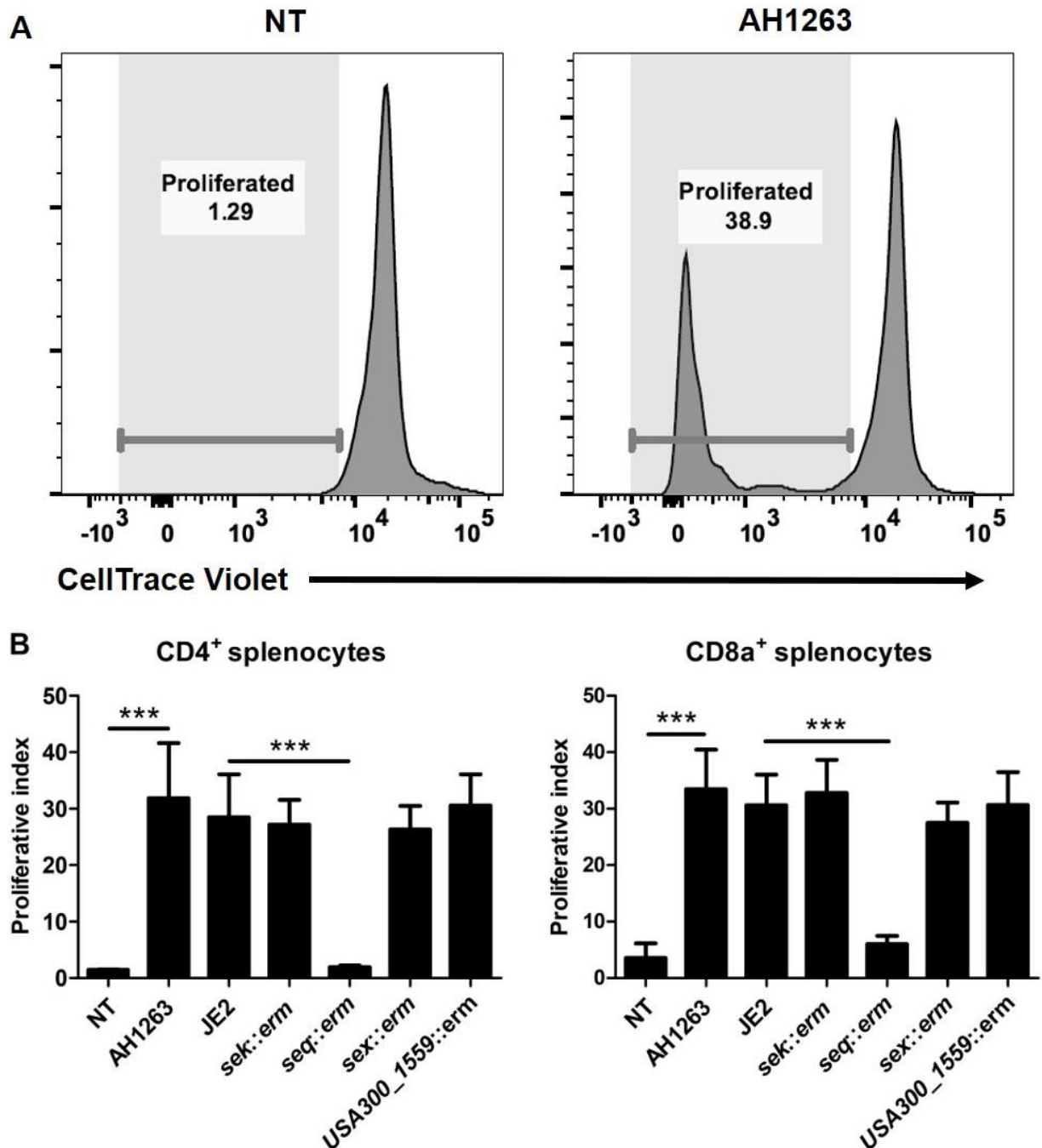
no difference in RNS production (**Figure 16**), suggesting that an increase in RNS production is likely not responsible for increased restriction of *S. aureus* growth *ex vivo*. Together, these data suggest that EPS reduces bacterial burden by inducing MΦs that restrict *S. aureus* growth through ROS.

### **Effect of EPS-Induced Macrophages on *S. aureus* Superantigen-Induced Activation of T Cells**

EPS was previously demonstrated to induce anti-inflammatory M2 MΦs that limit T cell activation (Paynich et al., 2017). Here, we found that these MΦs restrict *S. aureus* growth through ROS production, a feature classically associated with pro-inflammatory M1 MΦs. We reasoned that this apparent paradox could be explained by the flexible nature of MΦs, constantly modulating their functions according to environmental cues (Gautier et al., 2012; Murray et al., 1985). If this is the case, we hypothesized that EPS-induced M2 MΦs, when infected with *S. aureus*, may convert to pro-inflammatory M1 MΦs to deal with the bacterial infection. It is thought that M1 and M2 MΦ polarization is mutually exclusive because of the competition of the two M1- and M2-defining enzymes, iNOS and Arg-1, for the same substrate (Munder et al., 1998). To test this idea, we took advantage of the fact that *S. aureus* secretes both lipopeptides that trigger pro-inflammatory responses through TLR2 (Hashimoto et al., 2006) and SAGs that drive polyclonal activation of T cells (Peavy et al., 1970). We first tested a system for SAg-driven splenic T cell activation *in vitro* using culture supernatants from *S. aureus* AH1263. When C57BL/6J splenocytes were stimulated with 33% *S. aureus* AH1263 culture supernatants for 4 days, we found that both CD4<sup>+</sup> and CD8a<sup>+</sup> T cell proliferated, as measured by the dilution of CellTrace Violet proliferation tracking dye (**Figure 17**). *S. aureus* LAC makes three SAGs: SEI-K, SEI-Q, and SEI-X encoded by *sek*,

*seq*, and *sex*, respectively. It also harbors a gene annotated as a putative type A enterotoxin, *USA300\_1559*. We also stimulated the splenocytes with culture supernatants from transposon mutants deficient in each one of these SAgS and found that while the wild-type (JE2) stimulated proliferation of both CD4<sup>+</sup> and CD8a<sup>+</sup> splenocytes, mutant deficient in SEI-Q failed to stimulate proliferation (**Figure 17**).





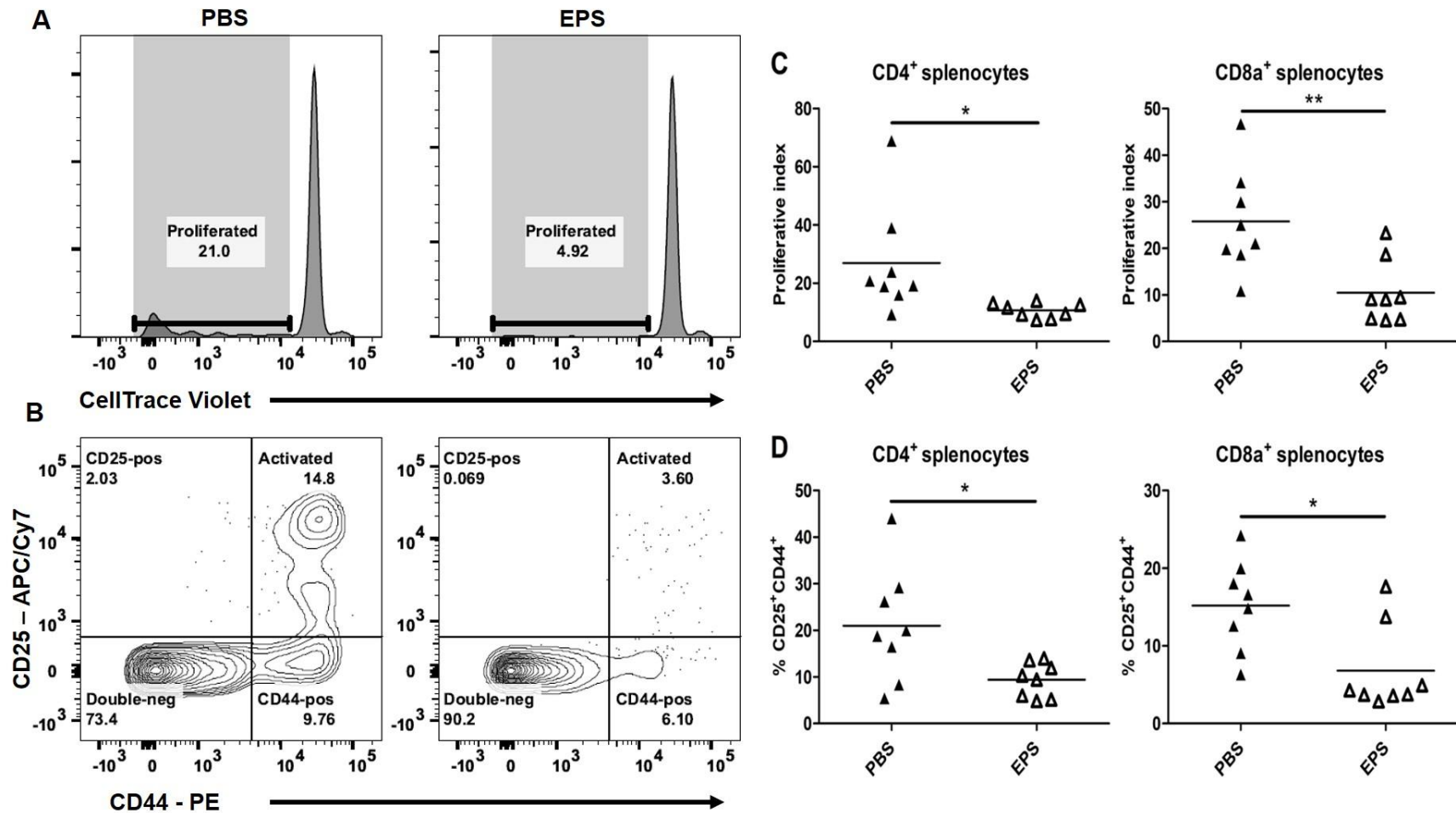
**Figure 17: Effect of *S. aureus* SAg on Splenic T Cell Proliferation *In Vitro*.**

Splenocytes ( $3.5 \times 10^5$ ) from mice were stimulated with 33% culture supernatant from *S. aureus* for 4 days. Proliferation of CD4<sup>+</sup> and CD8a<sup>+</sup> splenocytes were assessed by CTV dilution by flow cytometry. Proliferative index = % events within the “proliferated” gate.

**A.** Representative flow cytometry plots showing CD4<sup>+</sup> splenocytes. **B.** Error bars represent SD. Data pooled from 4 independent experiments. N = 4 (NT), 6 (all others).

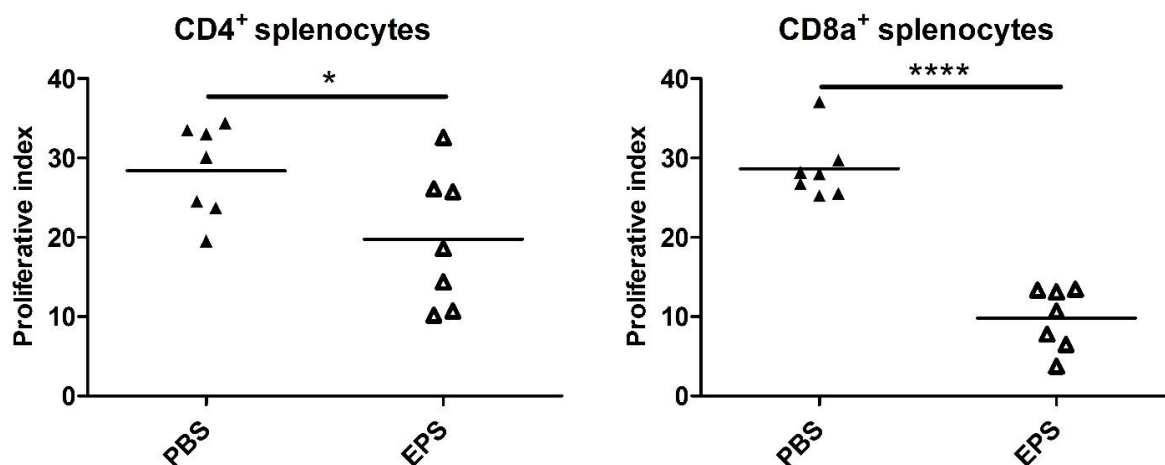
Data analyzed using 1-way ANOVA with Bonferroni’s multiple comparisons test.

\*\*\*P<0.001. Adopted from Paik and colleagues, *Infection and Immunity*, 2019.



**Figure 18: Effect of EPS-Induced Peritoneal MΦs on *S. aureus* SEI-Q-Mediated Proliferation and Activation of T Cells.** Splenocytes ( $3.5 \times 10^5$ ) from naïve mice were co-cultured with peritoneal F4/80<sup>+</sup> cells ( $5 \times 10^4$ ) from PBS- or EPS-treated mice 3 days after i.p. EPS. Cultures were stimulated with 33% *S. aureus* culture supernatant for 4 days. **A and B.** Representative flow cytometry plots showing CD8a<sup>+</sup> splenocytes. **C.** T cell proliferation. **D.** % CD25<sup>+</sup>CD44<sup>+</sup> activated T cells. Each triangle represents data from individual mouse. Bars represent mean. Data pooled from 3 independent experiments. N = 8. \*P<0.05. \*\*P<0.01. Adopted from Paik and colleagues, *Infection and Immunity*, 2019.

All other mutants tested caused proliferation of T cells (**Figure 17**), indicating that in the C57Bl/6J mouse background, *S. aureus* culture supernatants drive SEL-Q-dependent T cell proliferation. To test if EPS-induced MΦs retain their ability to inhibit T cell activation during *S. aureus* infection, we co-cultured F4/80<sup>+</sup> cells from PBS- or EPS-treated mice with splenocytes from naïve mice and stimulated them with *S. aureus* culture supernatant as in **Figure 17**. While co-culture with F4/80<sup>+</sup> cells from PBS-treated mice had proliferated and CD25<sup>+</sup>CD44<sup>+</sup> activated CD4<sup>+</sup> and CD8a<sup>+</sup> T cells, co-culture with F4/80<sup>+</sup> cells from EPS-treated mice had reduced T cell proliferation and activation (**Figure 18C and D**), suggesting that even in the presence of pro-inflammatory conditions, EPS-induced peritoneal MΦs retain the ability to limit T cell activation induced by *S. aureus* SEL-Q. To test if EPS also affects the activity of SAg *in vivo*, we stimulated splenocytes from EPS-treated mice 3 days after i.p. EPS injection with *S. aureus* culture supernatant *ex vivo*.

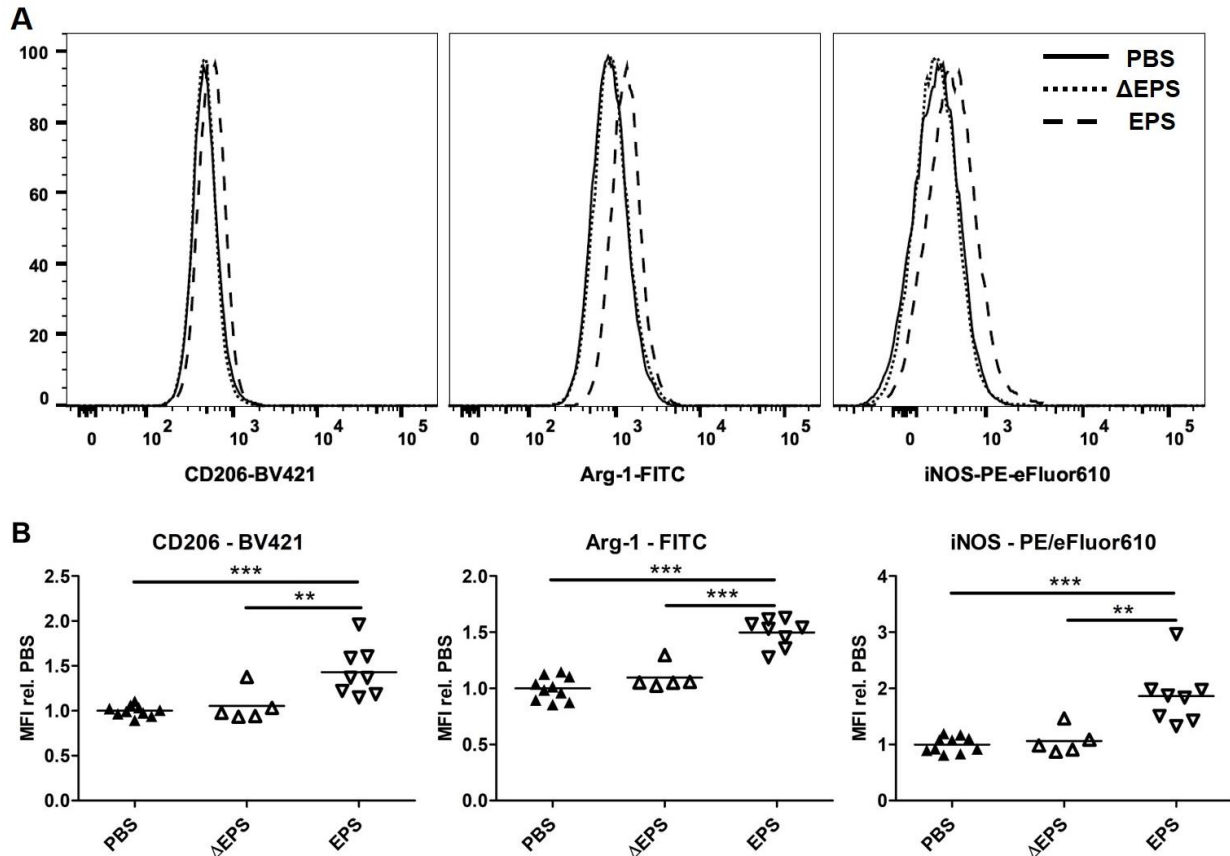


**Figure 19: Effect of EPS *In Vivo* on Splenic T Cell Proliferation Induced by *S. aureus* SEL-Q.** Mice were treated with PBS or EPS by i.p. injection and splenocytes were harvested 3 days later and labeled with CTV. CTV-labeled splenocytes were stimulated with 33% *S. aureus* culture supernatant for 4 days and assessed for proliferation by flow cytometry. Proliferative index = % events within “proliferated” gate. Each triangle represents data from 1 mouse. Bars represent mean. Data pooled from 3 independent experiments. N = 7. \*P<0.05. \*\*\*\*P<0.0001. Adopted from Paik and colleagues, *Infection and Immunity*, 2019.

We found that splenocytes from EPS-treated mice had reduced proliferation of both CD4<sup>+</sup> and CD8a<sup>+</sup> splenocytes (**Figure 19**), indicating that EPS limits the activation of T cells by SEI-Q *in vivo*. Together, these data suggest that EPS induced MΦs, while they have enhanced antimicrobial capacities like that of M1 MΦs, retain their ability to limit T cell activation like that of M2 MΦs.

### **Induction of Hybrid M1-M2 Macrophages by EPS**

MΦs have been classically described in two polarized states: pro-inflammatory M1 MΦs that mediate antibacterial immunity and anti-inflammatory M2 MΦs that mediate wound healing (Murray et al., 2014). EPS-induced peritoneal MΦs have previously been reported to be M2 MΦ (Paynich et al., 2017), but our data indicate that these MΦs also have enhanced antibacterial capacities like that of M1 MΦs. In reality, MΦs are flexible in nature, and their phenotypes depend on the signals they receive from the environment (Gautier et al., 2012; Murray et al., 2014). Recently, hybrid states of MΦs have been reported (O'Carroll et al., 2014). We hypothesized that EPS-induced MΦs might represent such a hybrid-type, displaying both M1- and M2-like qualities. To test this, we assessed the expression of the classical M1 marker, iNOS, and the M2 markers, CD206 and Arg-1, on EPS-induced MΦs. We found that, as previously reported, CD11b<sup>high</sup>F4/80<sup>high</sup> LPMs from EPS-treated mice had increased expression of CD206 and Arg-1 than LPMs from PBS- or ΔEPS-treated mice (**Figure 20**). In addition, LPMs from EPS-treated mice had increased expression of iNOS than LPMs from PBS- or ΔEPS-treated mice (**Figure 20**), indicating that the EPS-induced peritoneal MΦs are hybrid MΦs that express both M1 and M2 markers.



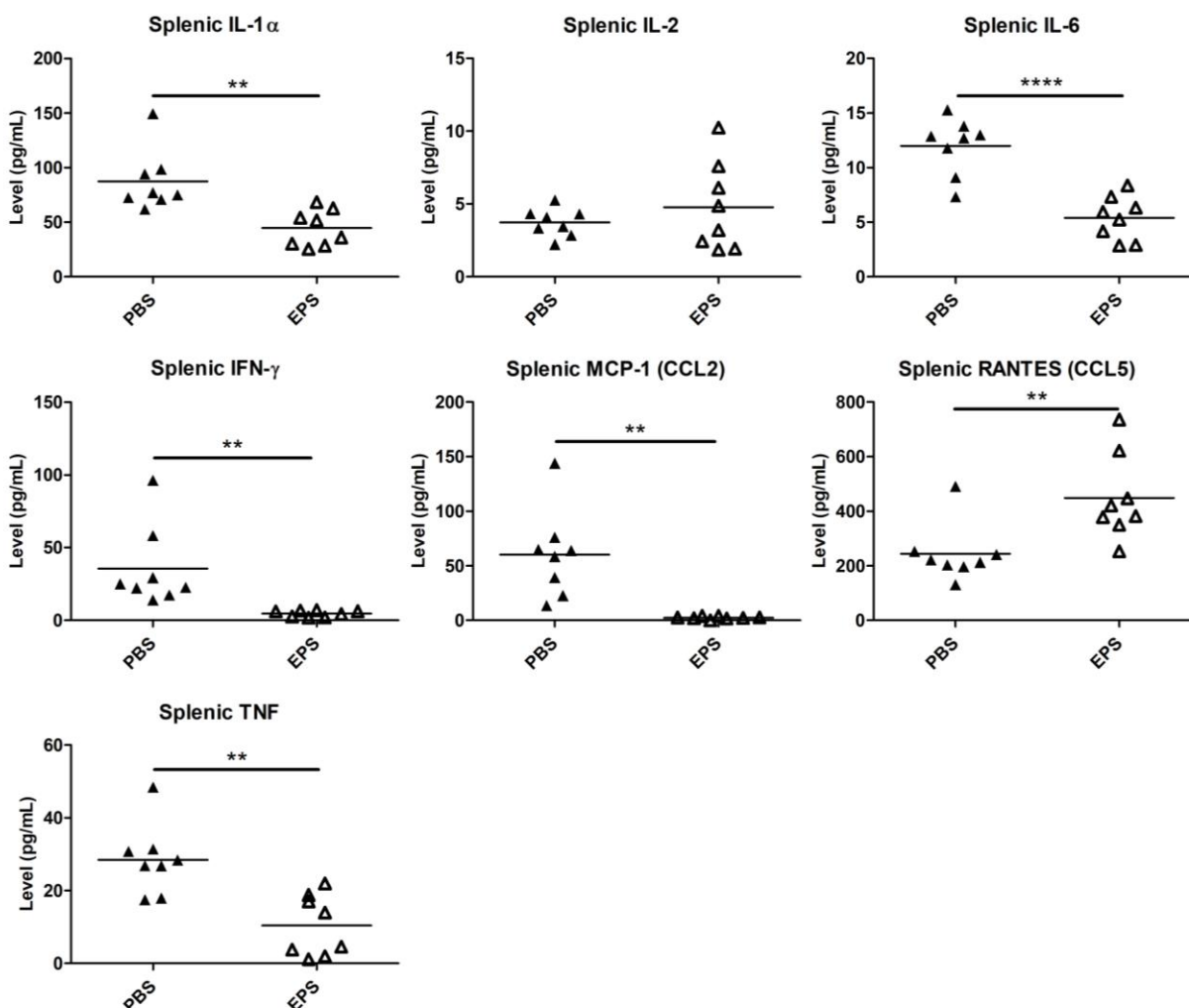
**Figure 20: Phenotype of EPS-Induced Peritoneal MΦs.** Peritoneal CD11b<sup>high</sup>F4/80<sup>high</sup> cells from PBS-, ΔEPS, and EPS-treated mice were analyzed 3 days after i.p. EPS injection by flow cytometry for M1 and M2 marker expression. **A.** Representative flow cytometry plots. Solid line (PBS), dotted line (ΔEPS), dashed line (EPS). **B.** MFI normalized to cells from PBS-treated mice. Each triangle represents data from one mouse. Data pooled from 3 independent experiments. N = 10 (PBS), 5 (ΔEPS), 8 (EPS). Data analyzed using 1-way ANOVA with Bonferroni's multiple comparisons test. \*\*P < 0.01. \*\*\*P < 0.001. Adopted from Paik and colleagues, *Infection and Immunity*, 2019.

### Effect of EPS on *S. aureus*-Induced Local Inflammation *In Vivo*

Upon entering the bloodstream, *S. aureus* disseminates to and seeds virtually all organs, setting off a complex cascade of events thought to lead to inflammation, tissue damage, and organ failure. We demonstrated that EPS might protect hosts from systemic *S. aureus* infection in part by MΦs that better control *S. aureus* growth and also limit inappropriate activation of T cells by *S. aureus* SAg, but many other mechanisms of protection may be at play given the complex pathophysiology of *S.*

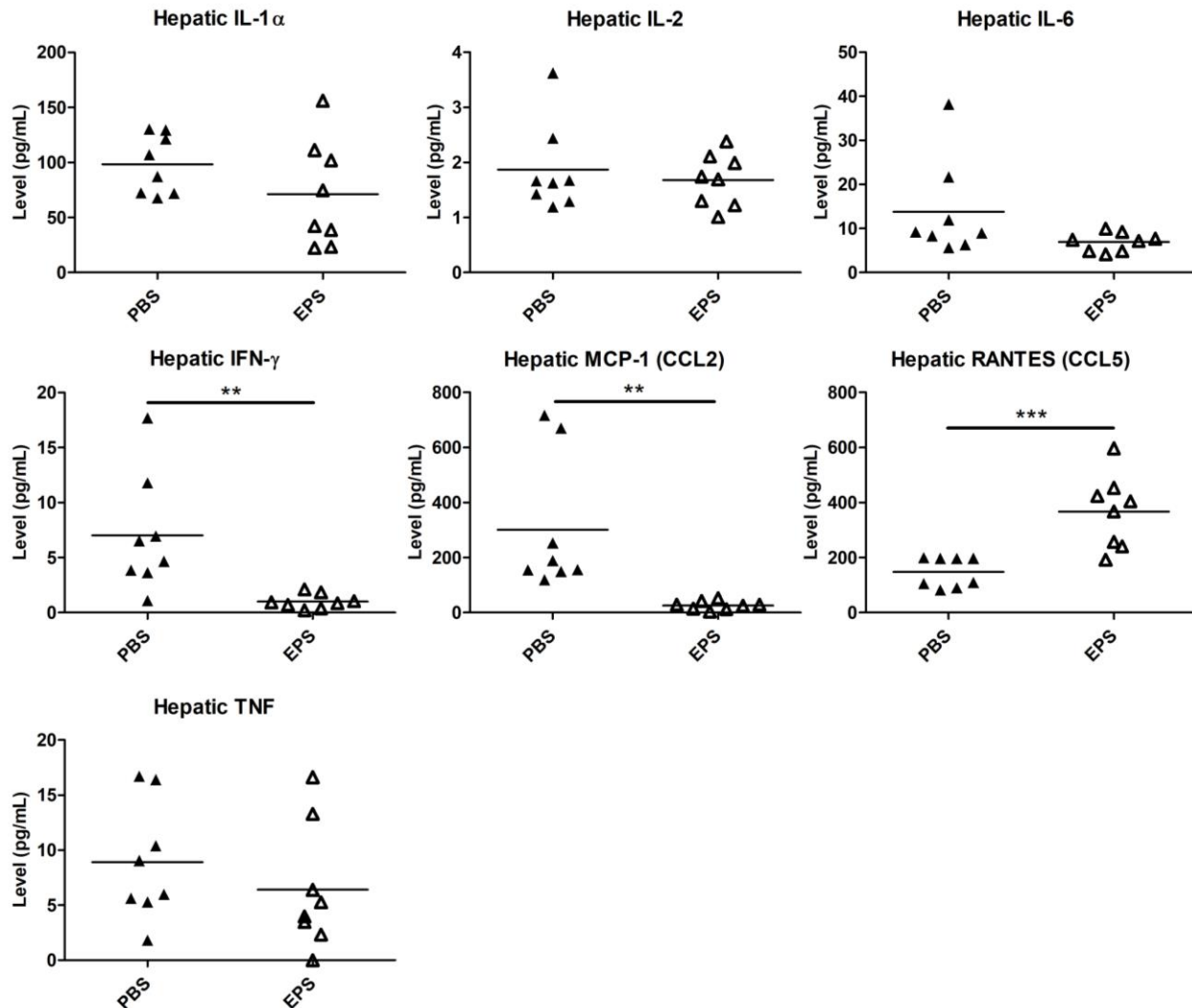
*aureus* bloodstream infection. We first focused on the observation that EPS-treated mice had reduced serum levels of IFN- $\gamma$  at 1 d.p.i. (**Figure 4**). IFN- $\gamma$  is the classic defining cytokine of type I immunity (Cher and Mosmann, 1987; Mosmann et al., 1986), and is associated with enhancing immunity against intracellular pathogens such as *L. monocytogenes* and *Mycobacterium tuberculosis* (Buchmeier and Schreiber, 1985; Flynn et al., 1993). Therefore, the observation that EPS-treated mice had reduced disease burden and reduced serum levels of IFN- $\gamma$  during *S. aureus* infection seemed paradoxical, given the apparent importance of IFN- $\gamma$  in antimicrobial immunity. However, neutralization of IFN- $\gamma$  during *S. aureus* bloodstream infection was reported to improve survival in mice (Nakane et al., 1995), and infection of IFN- $\gamma$ -deficient mice also results in improved survival compared to wild-type mice (Sasaki et al., 2000), suggesting that under certain circumstances including *S. aureus* bloodstream infection, IFN- $\gamma$  has a role in promoting pathogenesis. A significant producer of IFN- $\gamma$  is the T helper 1 (T<sub>H1</sub>) cell (Cher and Mosmann, 1987; Mosmann et al., 1986), and a major *S. aureus* factor that can stimulate T cells is SAgS (Peavy et al., 1970). Since EPS-induced M $\Phi$ s limit activation of T cells by SEL-Q *in vitro* (**Figure 18**), and splenocytes from EPS-treated mice proliferate less in response to SEL-Q (**Figure 19**), we hypothesized that a principal mechanism underlying protection by EPS in this model is the inhibition of IFN- $\gamma$  induced by *S. aureus* SAgS.

To assess the extent of the effects of EPS on limiting inflammation during *S. aureus* bloodstream infection *in vivo*, including the induction of IFN- $\gamma$ , we measured the levels of cytokines and chemokines in the organ homogenates from PBS- and EPS-treated mice. In the spleen, EPS-treated mice had reduced levels of IL-1 $\alpha$ , IL-6, IFN- $\gamma$ , MCP-1, and TNF, and increased levels of RANTES than PBS-treated mice (**Figure 21**).



**Figure 21: Effect of EPS on Splenic Cytokines and Chemokine Levels During *S. aureus* Bloodstream Infection at 1 d.p.i.** Levels of cytokines and chemokines were determined from spleen homogenates from PBS- or EPS-treated mice systemically infected with *S. aureus* at 1 d.p.i. by CBA. Each triangle represents data from one mouse. Bars represent mean. Data pooled from 2 independent experiments. N = 8. \*\*P < 0.01. \*\*\*\*P < 0.0001.

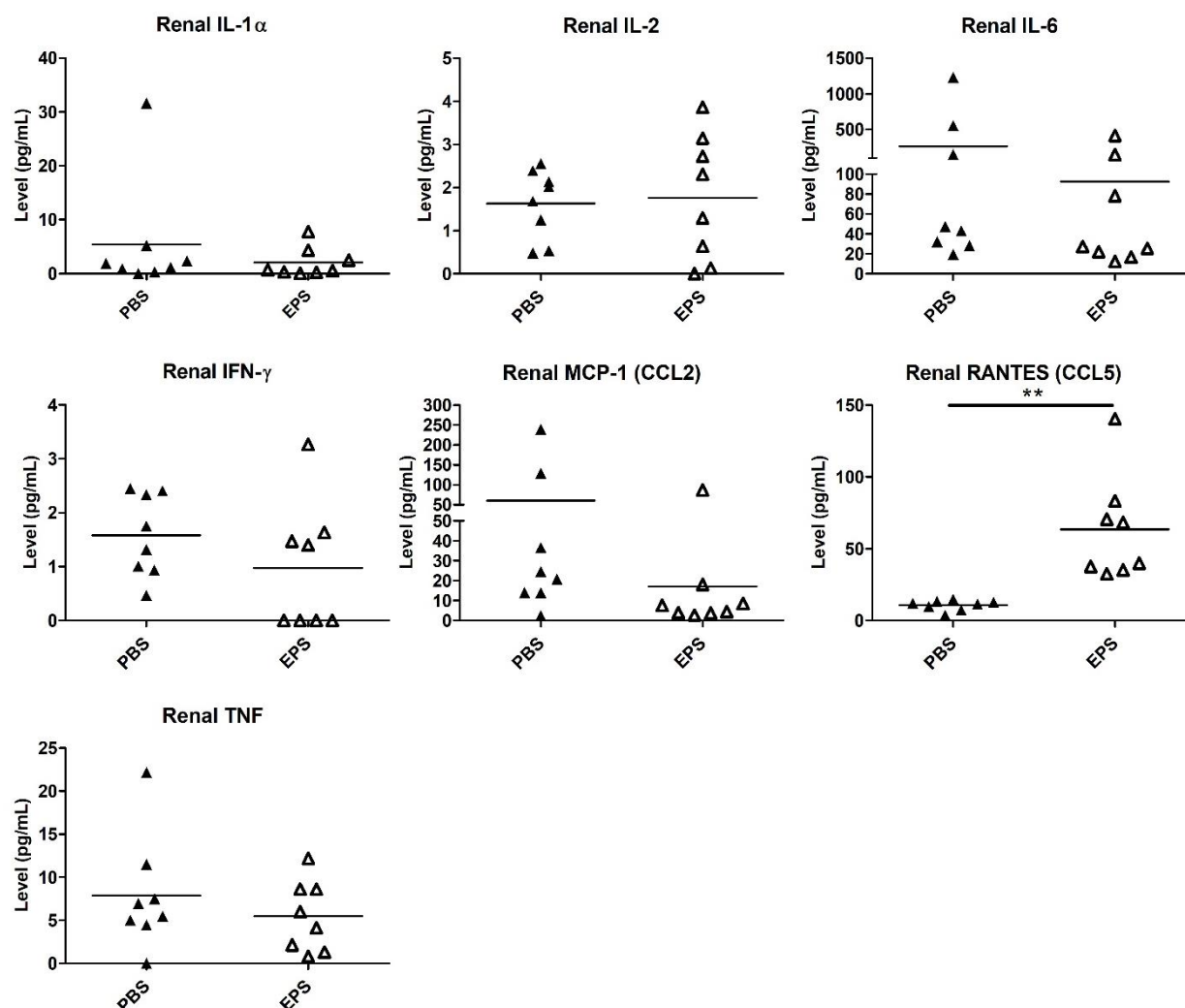
IL-2 levels were low in both PBS- and EPS-treated mice and no differences were observed between the two groups (**Figure 21**). In the liver, levels of IFN- $\gamma$  and MCP-1 were reduced in EPS-treated mice compared to PBS-treated mice while RANTES levels were increased (**Figure 22**). A small decrease, though not statistically significant, in IL-1 $\alpha$  levels were observed in the liver of EPS-treated mice compared to PBS-treated mice (**Figure 22**).



**Figure 22: Effect of EPS on Hepatic Cytokine and Chemokine Levels During *S. aureus* Bloodstream Infection at 1 d.p.i.** Levels of cytokines and chemokines were determined from liver homogenates from PBS- or EPS-treated mice systemically infected with *S. aureus* at 1 d.p.i. by CBA. Each triangle represents data from one mouse. Bars represent mean. Data pooled from 2 independent experiments. \*\* $P < 0.01$ . \*\*\* $P < 0.001$ .

No differences in IL-6 and TNF levels were observed between the two groups, and IL-2 levels were equally low in both groups. In the kidney, the only molecule showing a difference between the two groups was RANTES, which was increased in EPS-treated mice compared to PBS-treated mice (**Figure 23**).





**Figure 23: Effect of EPS on Renal Cytokines and Chemokines Levels During *S. aureus* Bloodstream Infection at 1 d.p.i.** Levels of cytokines and chemokines were determined from kidney homogenates from PBS- or EPS-treated mice systemically infected with *S. aureus* at 1 d.p.i. by CBA. Each triangle represents data from one mouse. Bars represent mean. Data pooled from 2 independent experiments. \*\*P<0.01.

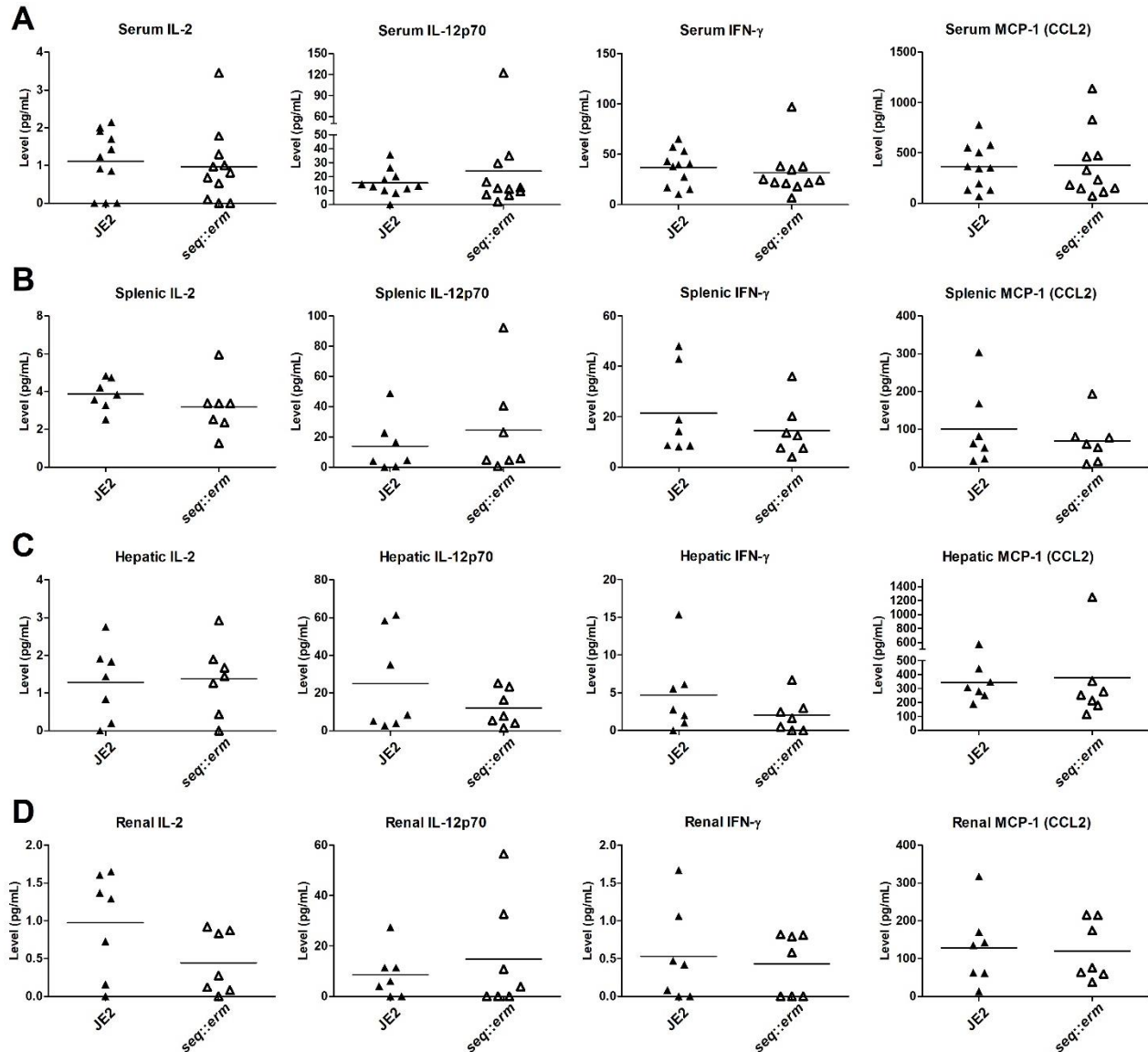
A small decrease in MCP-1 levels in EPS-treated mice compared to PBS-treated mice was observed, although it did not reach statistical significance (**Figure 23**). IFN- $\gamma$  and IL-2 levels in the kidney in both groups were deficient, and no differences were observed between the two groups (**Figure 23**). A much higher level of IL-6, compared to the spleen and liver, was observed in both groups in the kidney (**Figure 23**). Across the three organs, no differences in IL-1 $\beta$ , IL-10, IL-12p70, IL-17A, KC, MIP-1 $\alpha$ , MIP-1 $\beta$ , and

G-CSF were observed between PBS- and EPS-treated mice (**data not shown**).

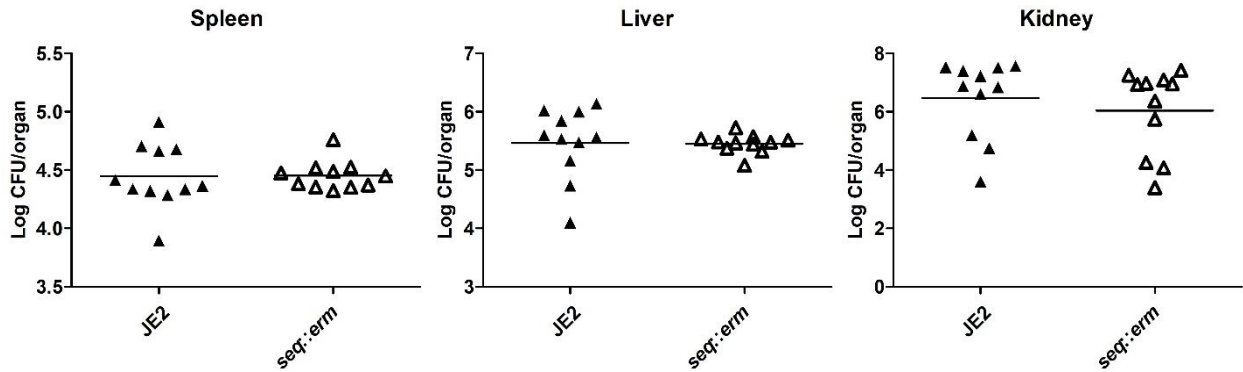
Together, these data suggest that EPS alters local inflammation during systemic *S. aureus* infection, reducing levels of cytokines and chemokines in the spleen and liver but not in the kidney. We also confirmed that EPS reduced IFN- $\gamma$  levels in the spleen and liver but not in the kidney.

### **Effect of Superantigen on the Pathogenesis of Systemic *S. aureus* Infection**

EPS limits the induction of IFN- $\gamma$  both systemically and locally during *S. aureus* infection, and IFN- $\gamma$ -deficient mice have improved survival upon systemic infection with *S. aureus* (Sasaki et al., 2000). *S. aureus* SAg are major virulence factors thought to drive polyclonal activation of T cells (Peavy et al., 1970), the significant producers of IFN- $\gamma$  (Cher and Mosmann, 1987; Mosmann et al., 1986). This ability to induce inappropriate activation of T cells have been the focus of studies on SAg (Bonventre et al., 1984), and the role of SAg in *S. aureus* pathogenesis have not been extensively studied. One study assessed the role of SAg using *S. aureus* Newman, that encodes SEA (Baba et al., 2008). In this study, Xu and colleagues infected mice C57Bl/6Cr1 (Charles River strain) mice with *sea*-deficient mutant *S. aureus* and found that levels of IFN- $\gamma$  and IL-12 were reduced in both in the serum and liver at 8 h.p.i. compared to mice infected with wild-type *S. aureus* (Xu et al., 2014). In addition, CFU *S. aureus* in the liver and heart, but not the kidney and the lung, were reduced in *sea* mutant infected mice compared to wild-type infected mice at 8 h.p.i. (Xu et al., 2014). We noticed that this pattern of reduced IFN- $\gamma$  levels early during infection, in the serum and the liver but not in the kidney, along with reduced bacterial burden in the liver but not in the kidney, was strikingly similar to that we observed in EPS-treated, *S. aureus*-LAC infected mice.



**Figure 24: Role of SEL-Q on Systemic and Local Cytokine and Chemokine Levels During *S. aureus* Bloodstream Infection at 1 d.p.i.** Mice were infected with wild-type (JE2) or SEL-Q-deficient mutant (*seq::erm*) *S. aureus* and levels of cytokines and chemokines in the serum (A) and spleen (B), liver (C), and kidney (D) homogenates collected on 1 d.p.i. were determined by CBA. Each triangle represents data from one mouse. Bars represent mean. Data pooled from 3 independent experiments. N = 11 (A), 7 (B-D).



**Figure 25: Role of SEL-Q on Bacterial Burden During *S. aureus* Bloodstream Infection at 1 d.p.i.** Mice were infected with wild-type (JE2) or SEL-Q-deficient (*seq::erm*) *S. aureus* and CFU *S. aureus* in organ homogenates were determined at 1 d.p.i. Each triangle represents data from one mouse. Data pooled from 3 independent experiments. N = 11.

We reasoned that given our hypothesis that EPS functions in part by limiting SAg-induced activation of T cells *in vivo*, the protective phenotype of EPS should be reproduced by infecting mice with a SAg-deficient mutant in our system as well.

We found that in the C57Bl/6J background, only SEL-Q is the active SAg at least under *in vitro* conditions (**Figure 17**), so we tested this idea by infecting C57Bl/6J mice with the JE2 strain (wild-type for the transposon library mutants) or the *seq*-deficient library mutant (*seq::erm*) we used previously. However, we found that the levels of IFN- $\gamma$  and IL-12p70 were not different in all tissues tested between JE2-infected and *seq::erm*-infected mice (**Figure 24**), inconsistent with the *sea*-deficient mutant as reported by Xu and colleagues (Xu et al., 2014). While EPS-treated mice had reduced levels of MCP-1 in the serum, spleen, and liver, the SEL-Q-deficient mutant-infected mice did not have reduced MCP-1 levels in all tissues tested compared to wild-type-infected mice (**Figure 24**). IL-2 levels were low in all tissues in both groups (**Figure 24**). We also assessed the bacterial load in the organ homogenates at 1 d.p.i. and found no difference in CFU *S. aureus* between wild-type- and SEL-Q-deficient mutant-infected mice in all tissues tested (**Figure 25**), another discrepancy compared to *sea* mutants described by Xu and

colleagues and EPS-treated mice that had reduced CFU *S. aureus* in the spleen and liver. Together, these data suggest that during infection with *S. aureus* LAC in the C57Bl/6J mouse background, SEL-Q does not drive IFN- $\gamma$  production and does not promote bacterial survival early during infection.

### **Superantigen-Independent Induction of Interferon Gamma by *S. aureus***

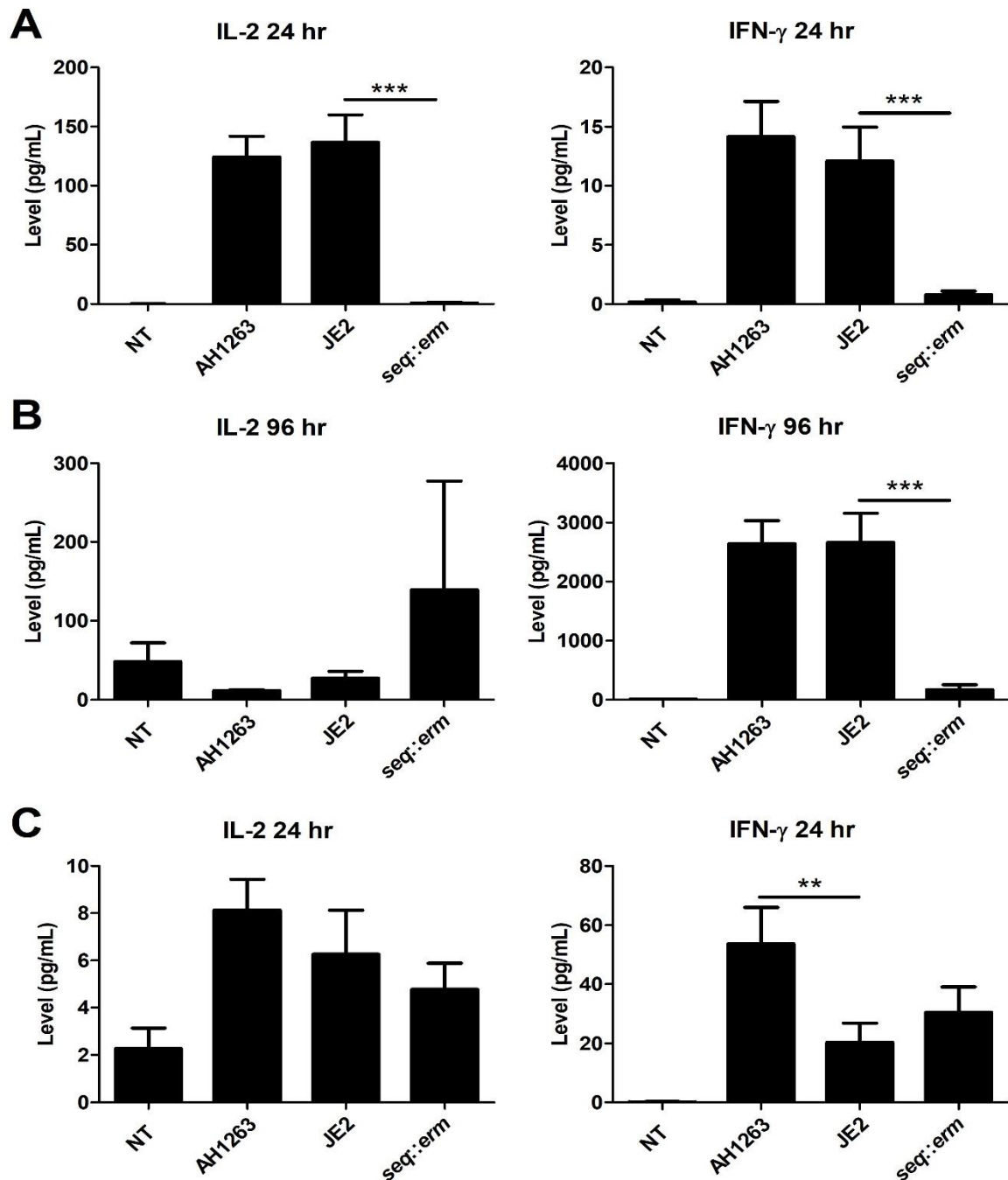
We hypothesized that two possibilities could explain why the SEL-Q-deficient mutant did not show reduced IFN- $\gamma$  production as predicted based on a past study on SEA (Xu et al., 2014). The first possibility stems from the differences in the two strains of *S. aureus* used between the SEA study and our study. *S. aureus* Newman encodes SEA and SEL-X (Baba et al., 2008; Wilson et al., 2011), but only SEA is responsible for driving T cell activation in this strain (Xu et al., 2014). In contrast, *S. aureus* LAC encodes SEL-K, -Q, and -X along with *USA300\_1559*, a putative type A enterotoxin, though only SEL-Q was responsible for T cell proliferation *in vitro* in the C57Bl/6J background (**Figure 17**). Still, the other three SAGs could be active *in vivo*, which would explain why the SEL-Q single-deficient mutant was able to induce IFN- $\gamma$  during infection (**Figure 24**). However, only SEL-Q had activity *in vitro* in *S. aureus* LAC just as only SEA had activity in *S. aureus* Newman, making it somewhat unlikely that the other SAGs could function *in vivo* at least in the C57Bl/6J background.

Another possibility is that *S. aureus* has a SAG-independent mechanism for inducing host IFN- $\gamma$  responses. One thing we noticed was that *in vivo*, IL-2 levels were not elevated during infection with *S. aureus* across our experiments. IL-2 is the T cell growth factor produced by T cells upon activation to support its proliferation (Mier and Gallo, 1980), so the lack of IL-2 elevation during infection at 1 d.p.i. seemed to suggest that the production of IFN- $\gamma$  at this point may not be driven by T cells. Another relevant

observation was made in a study that looked for endotoxin-induced IFN- $\gamma$  responses in mouse splenocytes. In this study, Varma and colleagues stimulated splenocytes from BALB/c mice *in vitro* with LPS and a few different heat-killed bacteria, including *S. aureus*, which resulted in the production of IFN- $\gamma$  (Varma et al., 2002). The way in which heat-killed *S. aureus* was prepared was not described by the authors, but SAGs are not expected to be produced by heat-killed *S. aureus*, though there may have been some residual SAGs in the preparation.

To confirm that heat-killed *S. aureus* can induce IFN- $\gamma$  from splenocytes, we isolated splenocytes from naïve C57Bl/6J mice and stimulated them with either 33% *S. aureus* culture supernatant as previously for SEI-Q-based stimulation, or heat-killed *S. aureus*, prepared after washing any secreted proteins away, at MOI 10 and measured the levels of cytokines and chemokines in the culture supernatants. At 24 hr, we found that cultures stimulated with *S. aureus* culture supernatant from AH1263 or JE2 had detectable IL-2 levels while the supernatant from SEI-Q deficient mutant did not (**Figure 26A**). Low levels of IFN- $\gamma$  were also detectable in the wild-type supernatant-stimulated cultures (**Figure 26A**), indicating that at 24 hr, *S. aureus* culture supernatant causes a SEI-Q-dependent induction of IL-2 and a little IFN- $\gamma$ , presumably from T cells. T cell proliferation induced by *S. aureus* culture supernatant can be detected by 4 days after stimulation *in vitro* (**Figure 17**), so we also measured cytokines from the supernatant-stimulated cultures at 96 hr. At this time point, high levels of IFN- $\gamma$  could be detected in cultures stimulated with the two wild-type *S. aureus* supernatants but not in the SEI-Q-deficient mutant (**Figure 26B**). At this time point, IL-2 levels in the stimulated wells did not differ from the untreated cultures (**Figure 26B**), indicating that IL-2 was no longer produced by 96 hr post stimulation. In contrast, the cultures

stimulated with heat-killed *S. aureus* produced IFN- $\gamma$  levels at 24 hr, and this effect was also observed using heat-killed SEI-Q-deficient mutant *S. aureus* (**Figure 26C**), indicating that SEI-Q was not required for IFN- $\gamma$  production in response to heat-killed *S. aureus*.



**Figure 26: Role of SEL-Q on *S. aureus* Culture Supernatant- or Heat-Killed *S. aureus*-Induced Stimulation of Mouse Splenocytes.** Splenocytes from mice were stimulated with 33% *S. aureus* culture supernatant (**A and B**) or heat-killed *S. aureus* at MOI 10 (**C**). Levels of cytokines in culture supernatant at 24 hr (**A and C**) and 96 hr (**B**) were assessed by CBA. Error bars represent SD. Representative data from 3 (**A and B**) or 4 (**C**) independent experiments. N = 3. Data assessed using 1-way ANOVA with Bonferroni's multiple comparisons test. \*\*P<0.01. \*\*\*P<0.001.

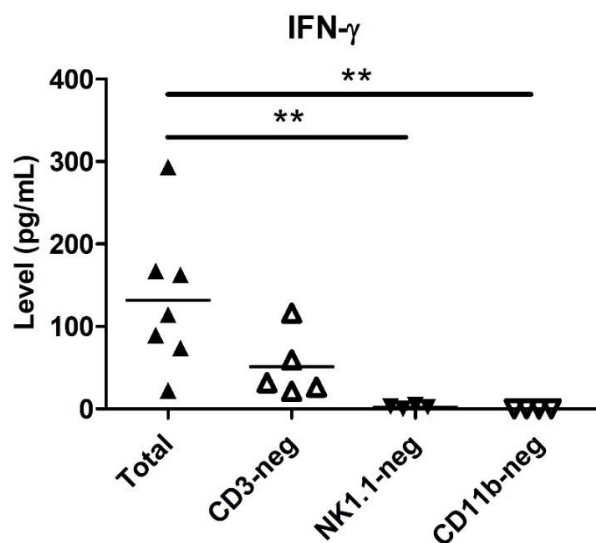
With the heat-killed *S. aureus*, a small increase in IL-2, although not statistically



significant, was observed with all strains tested (**Figure 26C**). Together, these data suggest that *S. aureus* harbors an alternative, SAg-independent mechanism to induce IFN- $\gamma$  from mouse splenocytes *in vitro*.

### Mechanism of SAg-Independent Induction of IFN- $\gamma$ by *S. aureus*

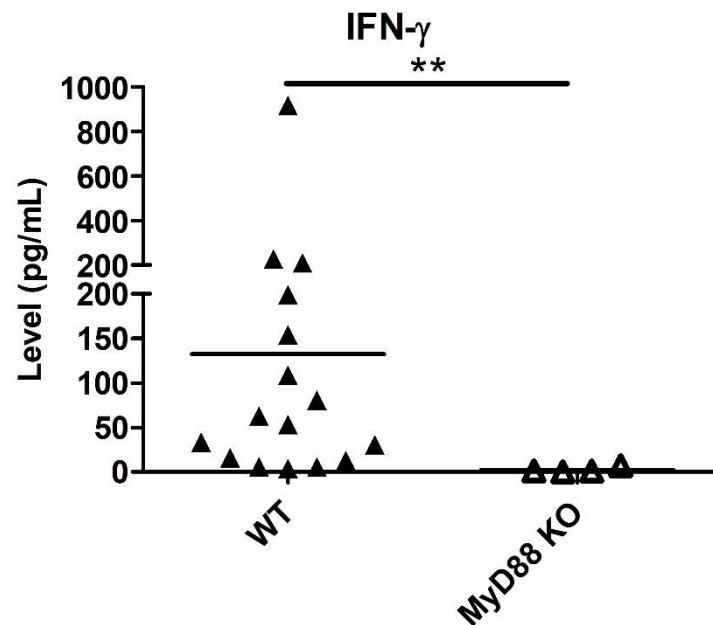
To elucidate the underlying mechanism by which heat-killed *S. aureus* induces IFN- $\gamma$ , we first tested for the cell types required for the production of IFN- $\gamma$  *in vitro*. The major producers of IFN- $\gamma$  that can be found in the spleen are T cells, NKT cells, NK cells, and myeloid cells, though the latter is considered a minor contribution (Emoto et al., 1999; Fukao et al., 2000; Mosmann et al., 1986; Scharon and Scott, 1993; Yeaman et al., 1998).



**Figure 27: Splenic Cell Types Required for the Induction of IFN- $\gamma$  by Heat-Killed *S. aureus*.** Total, CD3-, NK1.1-, or CD11b-depleted splenocytes from mice ( $3.5 \times 10^5$ ) were stimulated with heat-killed *S. aureus* at MOI 10 for 24 hr. IFN- $\gamma$  levels in culture supernatants were assessed by CBA. Each triangle represents data from one mouse. Bars represent mean. Data pooled from 4 independent experiments. N = 7 (total splenocytes), 5 (CD3-), 4 (other groups). Data were analyzed using 1-way ANOVA with Bonferroni's multiple comparisons test. \*\*P<0.01.

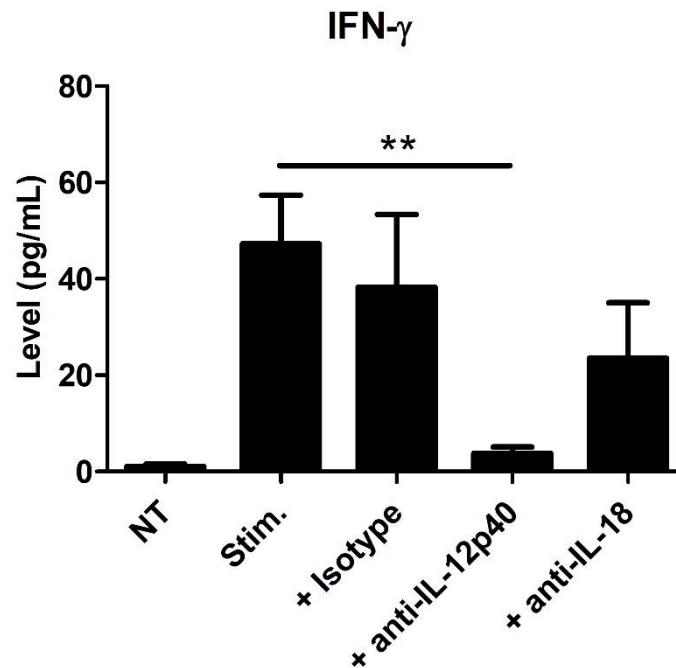
To test if these cells are required for heat-killed *S. aureus*-induced IFN- $\gamma$ , we depleted splenocytes of CD3<sup>+</sup> (T and NKT cells), NK1.1<sup>+</sup> (NK and NKT cells), or CD11b<sup>+</sup> (myeloid cells) cells and stimulated them with heat-killed *S. aureus in vitro*. We found that while depletion of CD3<sup>+</sup> cells resulted in only a minor reduction in IFN- $\gamma$  levels, depletion of NK1.1<sup>+</sup> or CD11b<sup>+</sup> cells completely abrogated IFN- $\gamma$  production (**Figure 27**), suggesting that NK cells and myeloid cells are required.

Given that NK cells are a typical IFN- $\gamma$ -producing cell type while myeloid cells are thought to be minor contributors (Fukao et al., 2000; Schariton and Scott, 1993; Yeaman et al., 1998), we reasoned that the myeloid cells might be playing a supportive role in NK cell-mediated production of IFN- $\gamma$ .



**Figure 28: Role of MyD88 on Induction of IFN- $\gamma$  by Heat-Killed *S. aureus*.** Splenocytes from MyD88-deficient mice were stimulated with heat-killed *S. aureus* at MOI 10 for 24 hr. IFN- $\gamma$  levels were assessed by CBA. Each triangle represents data from one mouse. Bars represent mean. Data for wild-type group was pooled from at least 6 independent experiments including historical data for comparison. MyD88 data pooled from 3 independent experiments. N = 4. Data analyzed using Mann Whitney U-test. \*\*P<0.01.

We hypothesized that for its supportive functions, myeloid cells would interact with *S. aureus* through its PRRs, mainly TLR2, to cause IFN- $\gamma$  production. To test this, we isolated splenocytes from MyD88-deficient mice and stimulated them with heat-killed *S. aureus*. Here, we found that the levels of IFN- $\gamma$  induced by heat-killed *S. aureus* were completely abrogated in MyD88-deficient mice compared to historic data on IFN- $\gamma$  induction in wild-type splenocytes (**Figure 28**), suggesting that recognition of *S. aureus* through PRRs was required.



**Figure 29: Role of IL-12 on Heat-Killed *S. aureus*-Induced IFN- $\gamma$  Production by Splenocytes.** Splenocytes from mice were stimulated with heat-killed *S. aureus* at MOI 10 in the presence of neutralizing anti-IL12p40, anti-IL-18, or isotype control antibodies for 24 hr. Levels of IFN- $\gamma$  in culture supernatants were determined by CBA. Error bars represent SD. Representative data from 3 independent experiments. N = 3. Data analyzed using 1-way ANOVA with Bonferroni's multiple comparisons test. \*\*P<0.01.

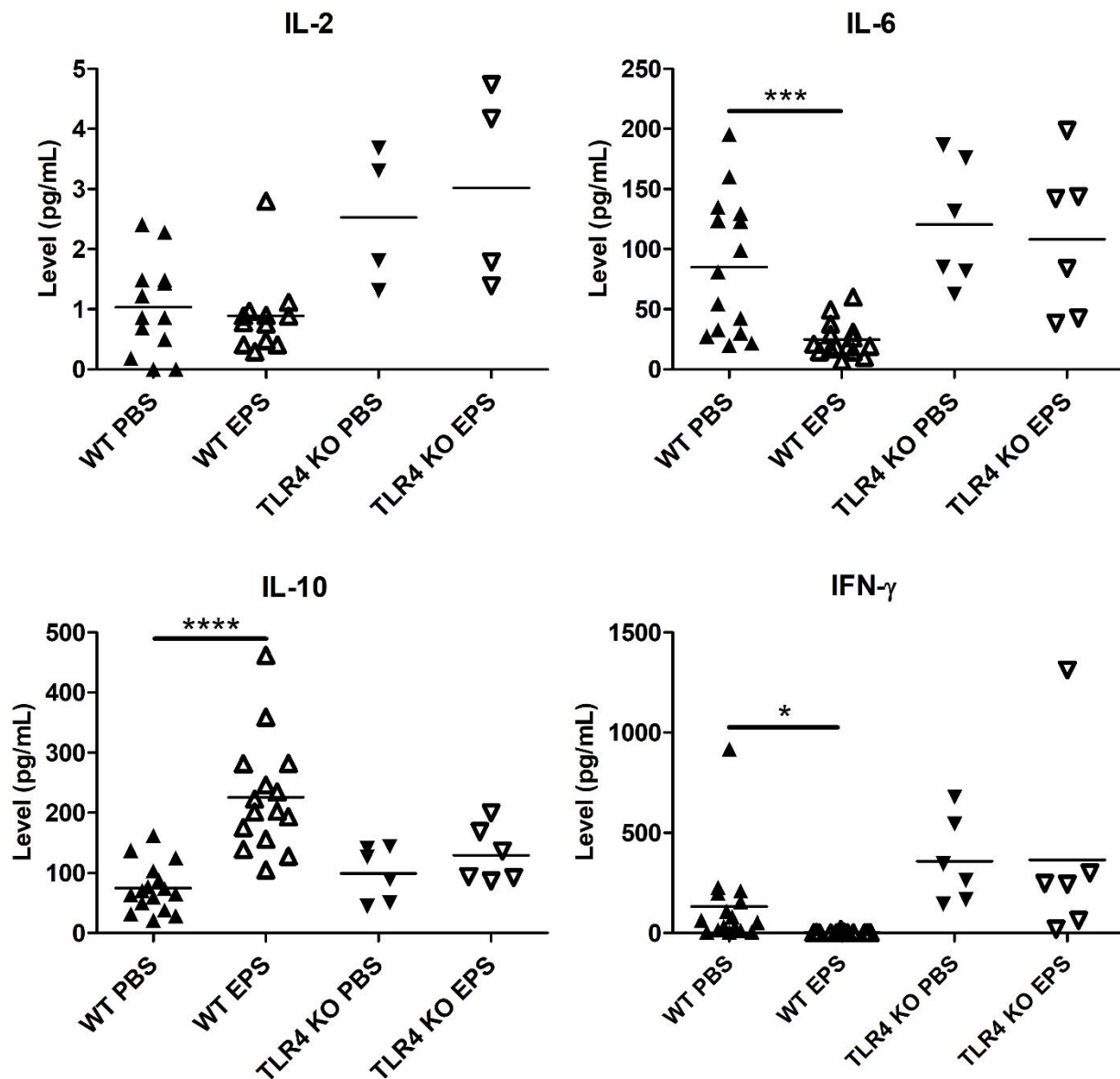
The finding that the induction of IFN- $\gamma$  requires both NK cells and myeloid cell activation by *S. aureus* through PRRs could result from cytokine-dependent activation of IFN- $\gamma$  production, as previously reported for NK cells (Chan et al., 1991). To test if

IFN- $\gamma$  induction by heat-killed *S. aureus* occurs through cytokine-dependent activation of NK cells, we neutralized the two known IFN- $\gamma$ -inducing cytokines IL-12 and IL-18 using antibodies when stimulating splenocytes with heat-killed *S. aureus*. We found that neutralization of IL-12p40 but not IL-18 or the addition of irrelevant antibody (isotype) reduced IFN- $\gamma$  levels in cells stimulated with heat-killed *S. aureus* (**Figure 29**).

Together, these data suggest that *S. aureus* can induce IFN- $\gamma$  through a SAg-independent mechanism, by activating NK cells to produce IFN- $\gamma$  through IL-12.

### **Effect of EPS on SAg-Independent Induction of IFN- $\gamma$ by *S. aureus***

Our data suggest that EPS reduces IFN- $\gamma$  levels during systemic *S. aureus* infection, and that this IFN- $\gamma$  is induced likely through both SAg-dependent and SAg-independent processes. This indicates that EPS interferes with IL-12-dependent activation of NK cells by *S. aureus*, but no evidence to date exists to support the idea that EPS affects NK cell functions. To test this, we treated mice with EPS by i.p. injection and isolated the total splenocytes the next day. We stimulated these splenocytes *ex vivo* with heat-killed *S. aureus* to see if EPS reduces NK cell-derived IFN- $\gamma$  production. While splenocytes from PBS-treated mice produced IFN- $\gamma$  in response to heat-killed *S. aureus*, splenocytes from EPS-treated mice essentially did not produce IFN- $\gamma$  (**Figure 30**). In addition, splenocytes from EPS-treated mice produced reduced levels of the pro-inflammatory cytokine IL-6 and higher levels of the anti-inflammatory cytokine IL-10 compared to cells from PBS-treated mice (**Figure 30**). No differences in IL-2 production were observed (**Figure 30**).



**Figure 30: Effect of EPS on Heat-Killed *S. aureus*-Induced Splenic Cytokine Response in Wild-Type and TLR4-Deficient Mice.** Splenocytes from PBS- or EPS-treated wild-type or TLR4 KO mice were stimulated with heat-killed *S. aureus* 1 day after i.p. EPS injection. At 24 hr, levels of cytokines in the culture supernatants were determined by CBA. Each triangle represents data from one mouse. Bars represent mean. Data pooled from 6 (WT) and 3 (TLR4 KO) independent experiments. N = 14-15 (WT), 4-6 (TLR4 KO). \* $P < 0.05$ . \*\*\* $P < 0.001$ . \*\*\*\* $P < 0.0001$ .

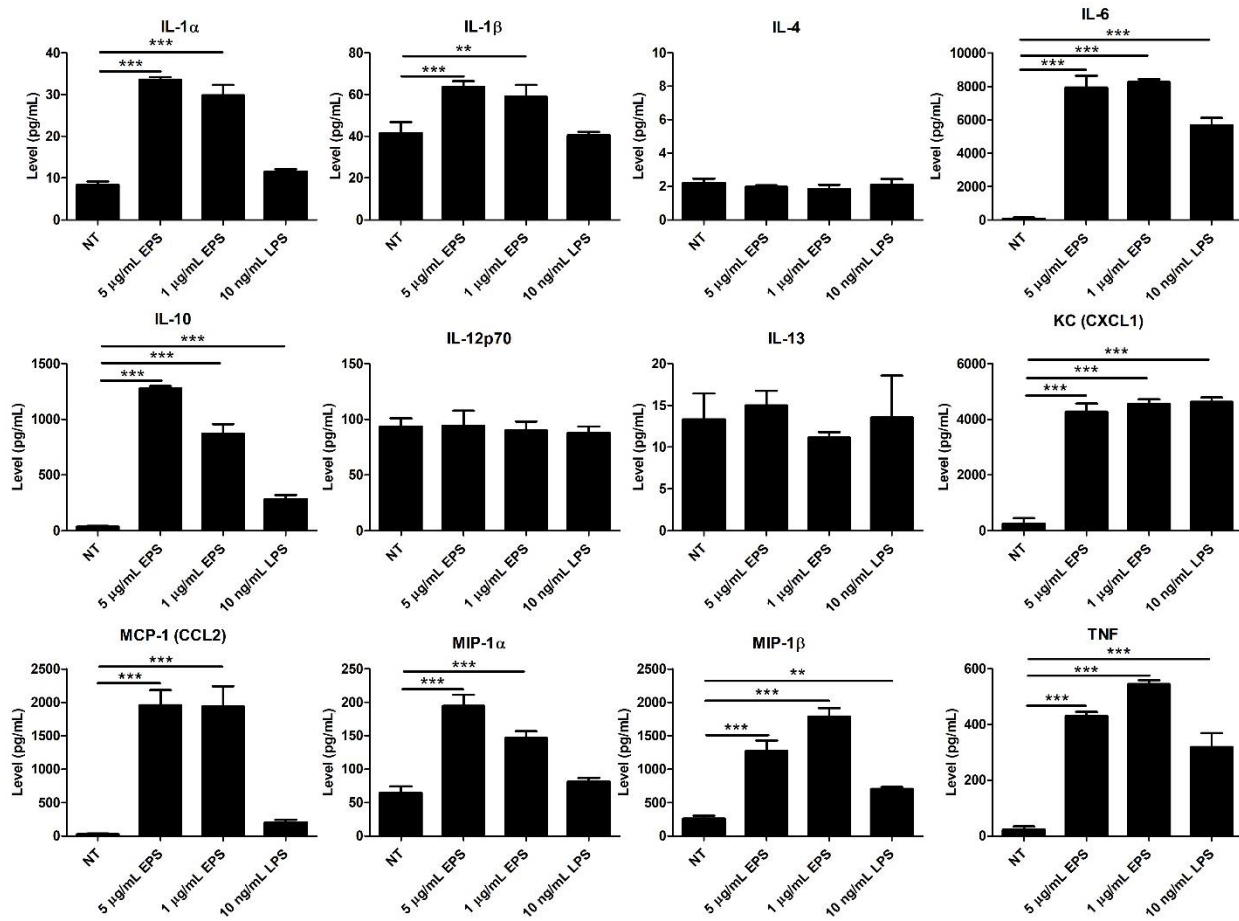
We then asked if this alteration of splenic cytokine response, especially the reduction in IFN- $\gamma$ , requires TLR4 by performing the same experiment with TLR4-deficient mice.

We found that in TLR4 KO mice, the reduction in IFN- $\gamma$  and IL-6, along with the

increase in IL-10 production by EPS in response to heat-killed *S. aureus* was abrogated (**Figure 30**). These data suggest that EPS reduces NK cell production of IFN- $\gamma$  in response to *S. aureus* through TLR4. In addition, EPS imprints an anti-inflammatory signature on splenic cytokine response to *S. aureus* through TLR4, reducing pro-inflammatory IL-6 production and increasing anti-inflammatory IL-10 production.

### **Immunostimulatory Properties of EPS**

EPS bolsters antimicrobial immunity and reduces inflammation by limiting SAg stimulation of T cells and by limiting IFN- $\gamma$  production by NK cells. This “EPS-primed” immunity results in reduced bacterial burden and limited inflammation during *S. aureus* bloodstream infection, improving host survival. However, the mechanism by which EPS is able to prime host immunity is not clear. To elucidate this mechanism, we sought to characterize the basic immunologic properties of EPS. One observation we focused on was that the effects of EPS, both from previous studies and from this study, required TLR4 (Jones et al., 2014; Paynich et al., 2017). TLR4 is best characterized as the signaling receptor for LPS, driving activation of NF- $\kappa$ B and subsequent production of pro-inflammatory mediators (Hoshino et al., 1999; Kawai et al., 1999). Given that the induction of peritoneal M2 M $\Phi$ s is TLR4-dependent (Paynich et al., 2017), we hypothesized that EPS could be activating TLR4 signaling in peritoneal M $\Phi$ s. To test this, we stimulated peritoneal F4/80<sup>+</sup> cells with EPS *in vitro* and measured the levels of cytokines and chemokines in the culture supernatant. We found that EPS treatment resulted in induction of many pro-inflammatory cytokines and chemokines as did LPS, including IL-6, KC, MIP-1 $\alpha$ , MIP-1 $\beta$ , and TNF (**Figure 31**).

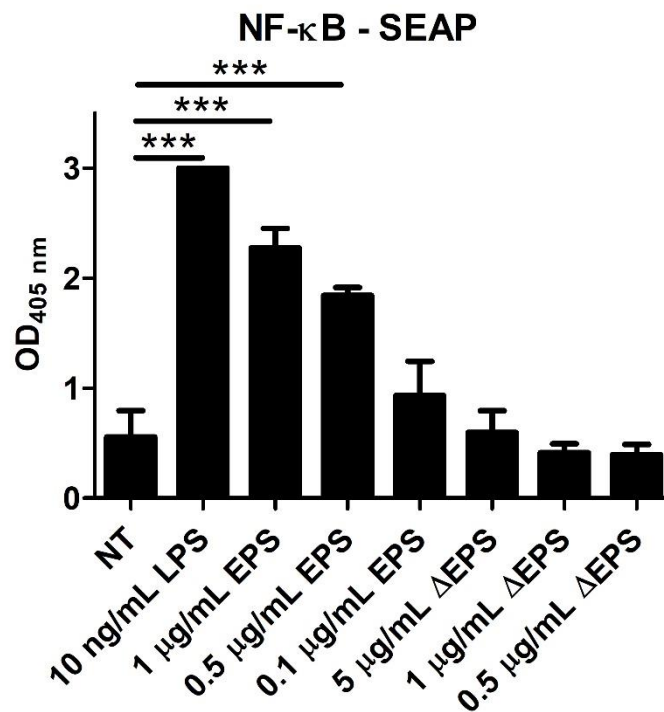


**Figure 31: Effect of EPS on Production of Cytokines and Chemokines by Peritoneal MΦs.** Peritoneal F4/80<sup>+</sup> cells (10<sup>5</sup>) from naïve Balb/cJ mice were stimulated with EPS or LPS and the levels of cytokines and chemokines in culture supernatants were measured at 24 hr using CBA. Error bars represent SD. Representative data from 4 independent experiments. N = 3. Data analyzed using 1-way ANOVA with Bonferroni's multiple comparisons test. \*\*P<0.01. \*\*\*P<0.001.

We also found that EPS stimulated the production of IL-1α, IL-1β, and MCP-1 while LPS failed to do so (**Figure 31**), at least at the concentrations we tested. We also observed that the anti-inflammatory cytokine IL-10 was produced by cells stimulated with EPS and LPS, though the levels were higher in EPS-treated cells (**Figure 31**). Paynich and colleagues previously reported that the peritoneal F4/80<sup>+</sup> cells increased intracellular levels of IL-4 and IL-13 upon stimulation using intracellular flow cytometry (Paynich et al., 2017), but we did not detect any increase in IL-4 or IL-13 levels in the supernatant of these cultures (**Figure 31**). We also observed that while EPS or LPS did not increase the

expression, some baseline level of IL-12p70 production by these cells was detected (**Figure 31**).

We next tested if EPS activates NF- $\kappa$ B activation. Paynich and colleagues previously reported that treatment of RAW 264.7 cells increases their expression of M2 M $\Phi$  markers(Paynich et al., 2017) , suggesting that EPS also activates RAW 264.7 cells. To test if EPS activates NF- $\kappa$ B, we utilized RAW 264.7 cells with a chromosomal integration of a secreted embryonic alkaline phosphatase (SEAP) gene whose expression is driven by NF- $\kappa$ B or activator protein 1 (AP-1), allowing estimation of NF- $\kappa$ B or AP-1 activation by measuring SEAP activity levels. We stimulated these reporter cells with EPS for 24 hr and measured the activity of SEAP in culture supernatants.



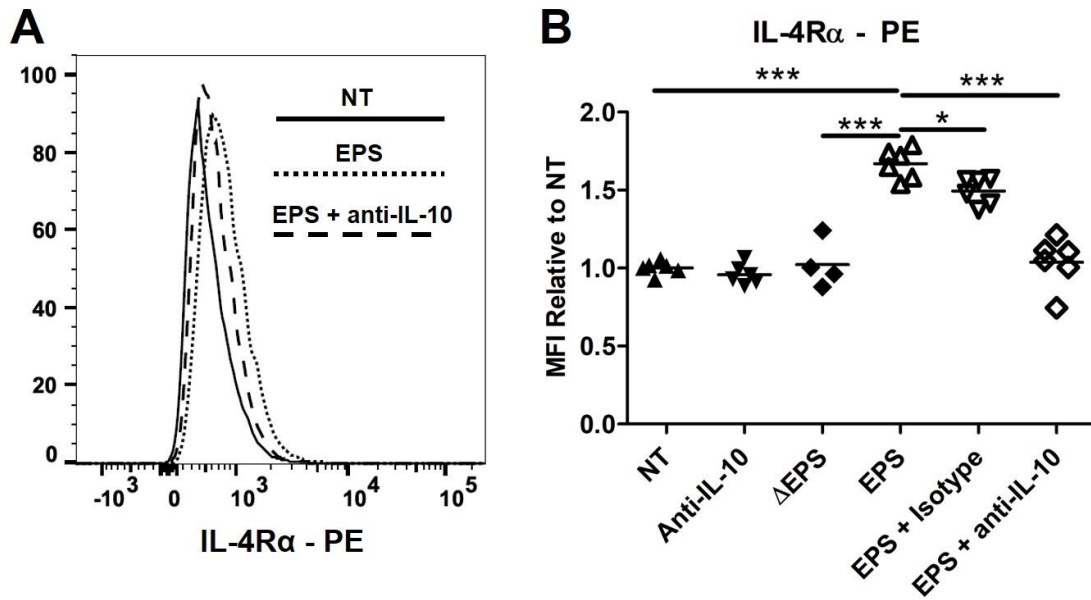
**Figure 32: Effect of EPS on NF- $\kappa$ B Activation.** RAW-SEAP cells were stimulated with either LPS, EPS, or  $\Delta$ EPS for 24 hr and the activity of NF- $\kappa$ B reporter, SEAP, was measured in culture supernatants using a colorimetric assay. Error bars represent SD. Representative data from 4 independent experiments. N = 3. Data analyzed using 1-way ANOVA with Dunnett's multiple comparisons test. \*\*\*P<0.001.



We found that like LPS, EPS induced SEAP activity from these cells in a dose-dependent manner (**Figure 32**), suggesting that EPS activates NF- $\kappa$ B.  $\Delta$ EPS was not able to induce NF- $\kappa$ B activity at any concentrations tested (**Figure 32**). Together, these data suggest that EPS harbors TLR agonistic properties *in vitro*.

### **Mechanism of EPS-Induced IL-4 Receptor Expression**

EPS induces the expression of M2 M $\Phi$  markers both *in vitro* and *in vivo* (Paynich et al., 2017). One key molecule associated with this effect has been the IL-4 receptor alpha chain (IL-4R $\alpha$ ), whose expression is increased in EPS-induced peritoneal LPMs and also in EPS-treated peritoneal M $\Phi$ s *in vitro* (Paynich et al., 2017). IL-4R $\alpha$  forms the IL-4R in complex with the common gamma chain ( $\gamma$ c) (Russell et al., 1993), and signaling through the IL-4R on M $\Phi$ s is the best characterized method for alternative activation (Stein et al., 1992). Two major cytokines have been demonstrated in the literature to increase the expression of IL-4R $\alpha$  on M $\Phi$ s, IL-6 and IL-10 (Hutchins et al., 2012; Mauer et al., 2014), both of which were stimulated from peritoneal M $\Phi$ s by EPS *in vitro* (**Figure 31**). To test which of these cytokines drives the increase in IL-4R $\alpha$  expression on M $\Phi$ s by EPS, we neutralized IL-6 and IL-10 during EPS stimulation of peritoneal cells using neutralizing antibodies. In a preliminary experiment, we found that neutralization of IL-6 did not affect the increase in IL-4R $\alpha$  expression by peritoneal CD11b<sup>high</sup>F4/80<sup>high</sup> LPMs *in vitro* (**data not shown**). However, in the presence of neutralizing anti-IL-10 antibodies, EPS did not increase IL-4R $\alpha$  expression by LPMs (**Figure 33**), indicating that the EPS-driven increase in expression of IL-4R $\alpha$  and *in vitro* initiation of the M2 polarization requires IL-10.



**Figure 33: Requirement for IL-10 on EPS-Induced Expression of IL-4Rα by Peritoneal MΦs.** Total peritoneal cells ( $3 \times 10^5$ ) were stimulated with EPS or  $\Delta$ EPS in the presence or absence of neutralizing anti-IL-10 antibody or isotype control. Expression of IL-4Rα on CD11b<sup>high</sup>F4/80<sup>high</sup> cells was assessed 24 hr later by flow cytometry. **A.** Representative flow cytometry data. **B.** Each symbol represents data from one mouse. Bars represent mean. Data pooled from 3 independent experiments. N = 4 ( $\Delta$ EPS), 6 (all others). Data analyzed using 1-way ANOVA with Bonferroni's multiple comparisons test. \* $P < 0.05$ . \*\*\* $P < 0.001$ .

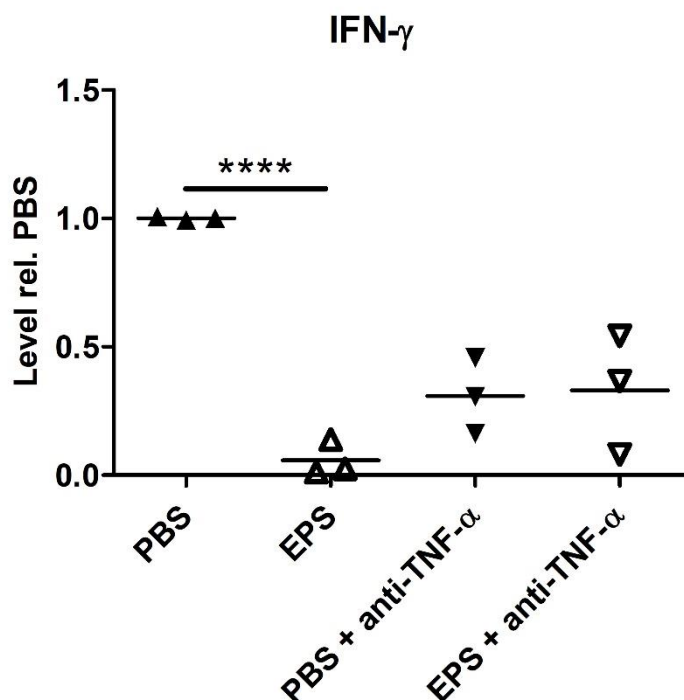
Addition of irrelevant antibody (isotype) to EPS-treated cultures resulted in only a small reduction in IL-4Rα expression (**Figure 33**).

### Mechanism of EPS-Induced Suppression of NK cell IFN-γ Response to *S. aureus*

We also sought to elucidate the underlying mechanisms by which EPS limits IFN-γ production during *S. aureus* infection. Paynich and colleagues had previously reported that EPS-induced M2 MΦs inhibit T cell activation through TGF-β and PD-L1 (Paynich et al., 2017). However, the mechanism by which EPS limits IFN-γ induced by heat-killed *S. aureus*, which we found is driven by cytokine-dependent activation of NK cells, is not clear. Given that TLR4 was required for EPS inhibition of NK cell IFN-γ

response to heat-killed *S. aureus*, we reasoned that some of the effector molecules EPS induces through TLR4 activation could also be required for this inhibition. We noticed that one cytokine induced by EPS treatment of peritoneal MΦs was IL-10 (**Figure 31**), an anti-inflammatory cytokine discovered because of its ability to inhibit IFN-γ production in T cells (Fiorentino et al., 1989). We also noticed that splenocytes from EPS-treated mice produced increased levels of IL-10 when stimulated with heat-killed *S. aureus* (**Figure 30**). We tested if EPS requires IL-10 to suppress IFN-γ responses to heat-killed *S. aureus* by administering neutralizing anti-IL-10 antibodies to mice one day prior to EPS treatment. Our preliminary results showed that splenocytes from mice treated with both EPS and the neutralizing anti-IL-10 antibody had reduced IFN-γ levels just like splenocytes from EPS-treated mice (**data not shown**), suggesting that IL-10 was not likely the suppressive factor.

We then sought for other potential mediators by which EPS limits IFN-γ production and focused on TNF-α that was also induced by EPS (**Figure 31**). TNF-α was originally discovered as a factor induced in serum after stimulation with endotoxin (Aggarwal et al., 1985; Carswell et al., 1975). TNF-α is generally associated with potent pro-inflammatory activity (Osborn et al., 1989b), and is a clinically relevant target in many inflammatory diseases. However, TNF-α has also been implicated in immunosuppression, especially against T cells (Speiser et al., 1996), and its activity is recognized to be more complex.

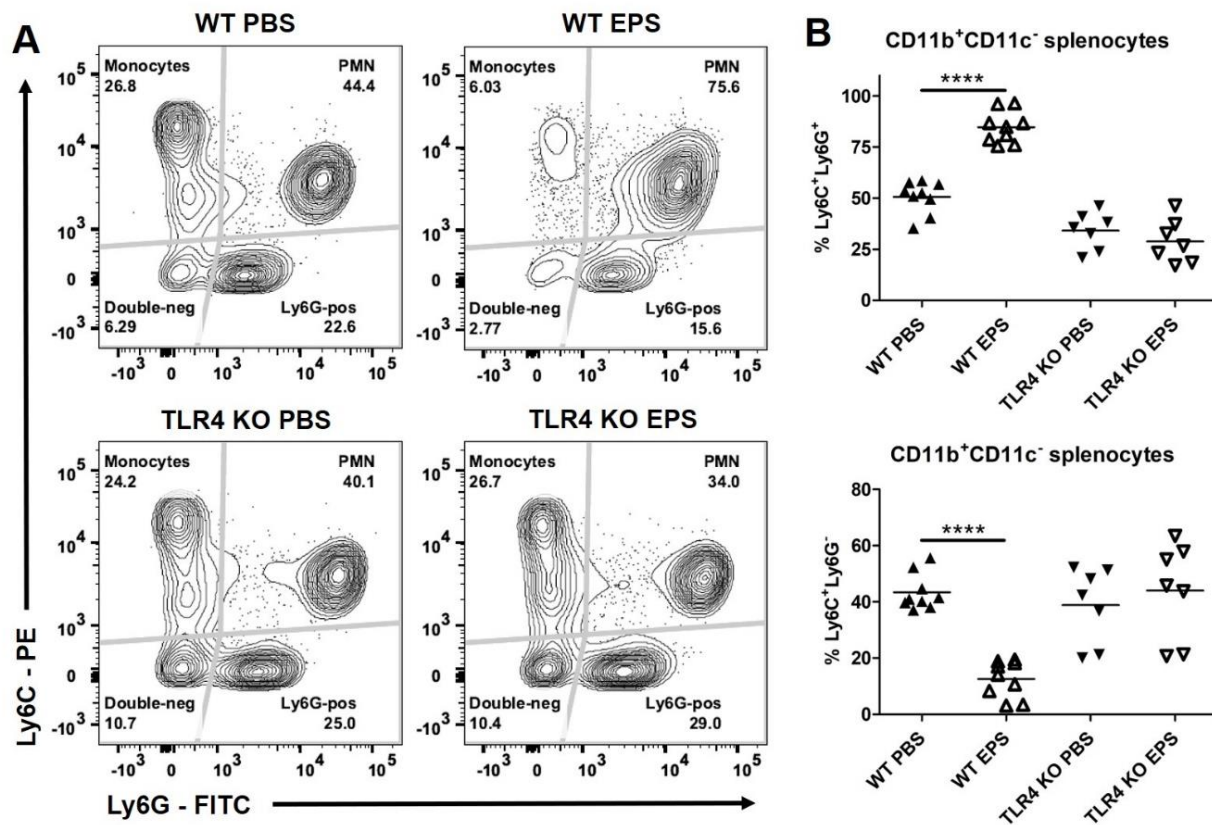


**Figure 34: Requirement of TNF- $\alpha$  for EPS Inhibition of IFN- $\gamma$  Induced by Heat-Killed *S. aureus*.** Mice were treated with neutralizing anti-TNF- $\alpha$  antibody 1 day prior to i.p. treatment with PBS or EPS. At 24 hr, splenocytes were harvested and stimulated with heat-killed *S. aureus* at MOI 10. Levels of IFN- $\gamma$  were determined from culture supernatants at 24 hr by CBA. Each triangle represents data from one mouse. Bars represent mean. Data pooled from 2 independent experiments. N = 3. \*\*\*\*P<0.0001.

To test if TNF- $\alpha$  was required for the inhibitory effects of EPS on heat-killed *S. aureus*-induced IFN- $\gamma$ , we administered neutralizing anti-TNF- $\alpha$  antibody to mice 1 day prior to EPS treatment. Splenocytes from these mice were stimulated with heat-killed *S. aureus*, and the levels of IFN- $\gamma$  were measured in the culture supernatants 24 hr later. While cells from EPS-treated mice produced less IFN- $\gamma$  compared to PBS-treated mice as we observed before, cells from EPS- and anti-TNF- $\alpha$  antibody treated mice did not produce less IFN- $\gamma$  than PBS- and anti-TNF- $\alpha$ -treated mice (**Figure 34**), suggesting that EPS limits IFN- $\gamma$  induced by heat-killed *S. aureus*, driven by NK cells, through TNF- $\alpha$ .

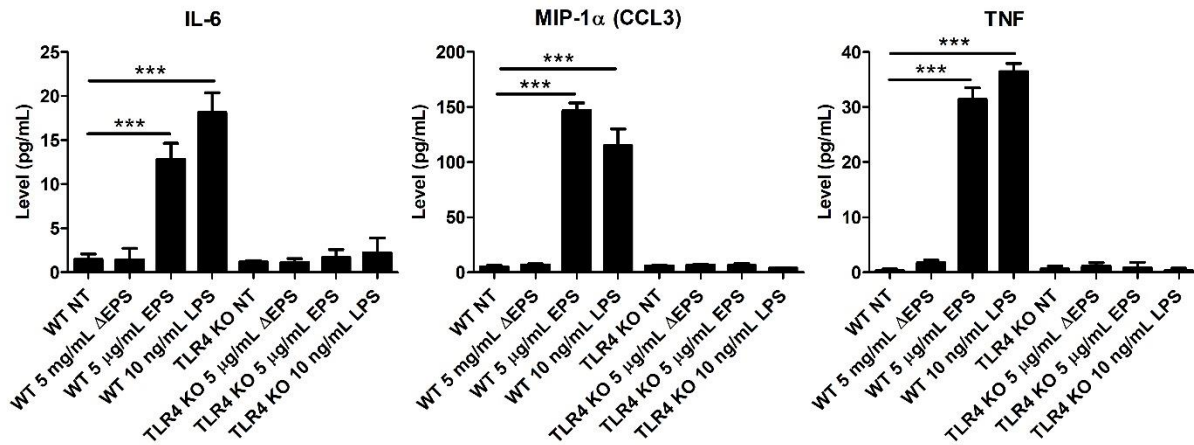
## Recruitment and Activation of Immune Cells by EPS

Another aspect of EPS we noticed was that EPS-treated peritoneal MΦs produced high levels of chemokines KC, MCP-1, MIP-1 $\alpha$ , and MIP-1 $\beta$  (**Figure 31**). Chemokines are known to be responsible for the recruitment of various immune cells to the tissue (Anisowicz et al., 1987; Martins-Green and Feugate, 1998), and since EPS induces the production of chemokines, we hypothesized that EPS could promote immune cell recruitment into tissues *in vivo*.



**Figure 35: Effect of EPS on Neutrophil Recruitment to the Spleen.** WT or TLR4-deficient mice were treated i.p. with PBS or EPS. The splenic myeloid compartment was assessed by flow cytometry 1 day later. **A.** Representative flow cytometry plots showing CD11b<sup>+</sup>CD11c<sup>-</sup> splenic myeloid compartment. **B.** Pooled quantification of % Ly6C<sup>+</sup>Ly6G<sup>+</sup> PMNs and % Ly6C<sup>+</sup>Ly6G<sup>-</sup> monocytes from the CD11b<sup>+</sup>CD11c<sup>-</sup> compartment. Each triangle represents data from one mouse. Bars represent mean. Data pooled from 4 independent experiments. N = 9 (WT), 7 (TLR4 KO). \*\*\*\*P<0.0001.

In fact, recruitment of CD11b<sup>+</sup>Ly6C<sup>+</sup>Ly6G<sup>-</sup> monocytes and CD11b<sup>+</sup>Ly6C<sup>+</sup>Ly6G<sup>+</sup> PMNs has been observed in the peritoneal cavity 1 day following i.p. EPS injection (Paynich, unpublished data). EPS-treated mice have reduced bacterial load in the spleen and liver during systemic *S. aureus* infection, so we tested if EPS can recruit immune cells to the spleen *in vivo* 1 day after i.p. EPS treatment. We found that while neutrophils represented about 50% of the splenic CD11b<sup>+</sup>CD11c<sup>-</sup> myeloid compartment in PBS-treated mice, more than 75% of this compartment was neutrophils in EPS-treated mice (**Figure 35**), suggesting that EPS induces neutrophil recruitment in the spleen. We also observed that while in PBS-treated mice 40% of the CD11b<sup>+</sup>CD11c<sup>-</sup> myeloid cells were Ly6C<sup>+</sup>Ly6G<sup>-</sup> monocytes, this compartment was reduced to about 15% in EPS-treated mice (**Figure 35**). We also tested if this recruitment depends on EPS activation of TLR4 using TLR4-deficient mice. We found that EPS-treated TLR4-deficient mice did not have increased percentages of Ly6C<sup>+</sup>Ly6G<sup>+</sup>PMNs compared to PBS-treated TLR4-deficient mice (**Figure 35**), suggesting that EPS recruits neutrophils to the tissue through TLR4 signaling. The reduction in % Ly6C<sup>+</sup>Ly6G<sup>-</sup> monocytes by EPS did not occur in TLR4-deficient mice as well (**Figure 35**).



**Figure 36: Effect of EPS on Cytokine and Chemokine Production by Neutrophils.** Bone marrow neutrophils ( $10^5$ ) isolated from WT or TLR4 KO mice were stimulated with 5  $\mu$ g/mL EPS or  $\Delta$ EPS, or 10 ng/mL LPS for 24 hr. Levels of cytokines and chemokines in culture supernatants were determined by CBA. Error bars represent SD. Representative data from 2 independent experiments. N = 3. Data analyzed using 1-way ANOVA with Bonferroni's multiple comparisons test. \*\*\*P<0.001.

Given that EPS activates MΦs to produce cytokines and chemokines (**Figure 31**), and that EPS recruits neutrophils to tissues (**Figure 35**), we wondered if EPS could also activate neutrophils for the production of cytokines and chemokines. We tested this by isolating bone marrow neutrophils (BM-PMNs) using gradient centrifugation and stimulating them with EPS *in vitro*. We found that stimulation with EPS and LPS, but not  $\Delta$ EPS, of BM-PMNs led to the production of IL-6, MIP-1 $\alpha$ , and TNF (**Figure 36**). In addition, production of these molecules required TLR4, as shown by the observation that treatment of TLR4-deficient BM-PMNs with EPS and LPS did not induce the production of these cytokines (**Figure 36**). Together, these data suggest that EPS can recruit and activate neutrophils through TLR4.

## CHAPTER FOUR

### DISCUSSION

#### **How Probiotics can Become Therapies**

##### **Barriers to Probiotic Therapy**

Microbes are gaining an appreciation for their ability to influence many host physiologic processes, especially the immune system. The use of microbes in order to benefit the host is a concept first characterized by Metchnikoff more than 100 years ago (Metchnikoff, 1908), and the practice of this is represented today by the large probiotic industry. It is relatively easy for the general public to access an array of probiotic products, but the majority of these products are food supplements, and only a limited number of probiotics have been approved for use in clinical settings for specific therapy. In 2010, the Panel on Dietetic Products, Nutrition and Allergies of the European Food Safety Authority gave negative opinions to all health claim submissions related to probiotics (Rijkers et al., 2011).

The reasons behind the difficulty of getting probiotics to the mainstream clinical settings are many and complex, including things such as the discrepancy in the field on how to measure health benefits. Still, the primary barrier is that the mechanistic basis for how probiotics, and microbes for that matter, confer health benefits are poorly understood. Especially concerning is the lack of knowledge on the molecular mediators that microbes produce to confer its benefits. Getting new drugs into the bedside relies on understanding their pharmacokinetic



properties, which allow for the prediction of drug efficacy, tissue distribution, adverse effects, and clearance; all are critical parameters required for clinicians to consider when tailoring their therapeutic approach to patients. Without identification of the molecules that microbes use to benefit hosts, characterization of the pharmacokinetic parameters of the probiotic is difficult, and so is predicting its efficacy and the adverse effects. There are reports of detrimental traits such as metabolic syndrome that can be conferred by the transfer of microbiota from one individual to another in animal models (Ridaura et al., 2013), demonstrating a need to understand the underlying mechanisms by which microbes affect host physiology.

However, this is not to downplay the prospect of microbial therapies. In fact, penicillin, the most famous and arguably the most successful medical therapy, is derived from a microbe (Fleming, 1929). Given the full range of physiologic systems that microbes are being demonstrated to affect, we suspect that studying microbes' probiotic benefits will reveal many novel therapeutic targets and agents that, if their mechanisms are elucidated, could be used to improve patient care in many disciplines.

In this study, we characterized the use of an EPS molecule derived from the probiotic bacterium, *B. subtilis*, in a model of systemic infection by *S. aureus*, to prevent hosts from succumbing to the disease. We found that EPS does this by stimulating the immune system through TLR4, which results in bolstering antimicrobial immunity while limiting specific aspects of host inflammatory processes, ultimately reducing disease burden and improving survival. However, the structure of EPS is yet unknown, and means to detect EPS reliably *in vivo* do not exist. Therefore, it is currently not possible to tract the levels of EPS after administration to hosts *in vivo*, preventing studies from elucidating its pharmacokinetic properties. Developing the tools to reliably

detect EPS *in vivo* would significantly advance our goal of developing EPS into a novel therapy for clinical use, since we will be able to understand better the tissue distribution of EPS, which will help us predict how EPS affects different host tissue sites, and clearance of EPS from the hosts, which will help avoid potential adverse effects of EPS.

### **Clinical Considerations for EPS Therapy in Sepsis**

We demonstrated that pre-treatment of mice with EPS improved outcomes from *S. aureus* bloodstream infection that occurred one day later. In the *C. rodentium*-induced colitis model, Jones and colleagues demonstrated that *B. subtilis* spores could be administered to mice as late as 3 days post exposure to *C. rodentium* (Jones and colleagues, unpublished data), suggesting that *B. subtilis* could potentially be used as a treatment for infectious colitis. However, we did not test if EPS could be administered to mice after exposure to *S. aureus* for protection. In this model, EPS-treated mice had signs of protection that could be detected as early as 6 h.p.i. (**Figure 6C**), and immune cells isolated from EPS-treated mice respond differently to *S. aureus ex vivo* by 1 day after EPS treatment (**Figure 10 and 30**), suggesting that EPS primes the host immunity in a way better suited for survival when challenged by *S. aureus*. In addition, the peak disease symptoms occur 10 d.p.i. in *C. rodentium*-induced colitis, so the administration of *B. subtilis* spores at 3 d.p.i. in this is still prior to the development of disease symptoms. Sepsis patients often present to the healthcare setting after the development of symptoms which rapidly deteriorate, making it a challenge for clinicians to identify patients rapidly and to immediately treat them (Rhodes et al., 2017; Singer et al., 2016). Therefore, testing if EPS could be used as a treatment for patients with an established bloodstream infection is clinically relevant.

Still, the pre-treatment model can be a successful therapy in many situations regarding sepsis. One major risk factor for developing bloodstream infections is invasive procedures. *S. aureus* is a major concern for these patients since *S. aureus* is normally found on patients on the skin and anterior nares (Gorwitz et al., 2008). During invasive procedures, *S. aureus* on the skin can get access into the bloodstream, a concern that is managed by antimicrobial prophylaxis (Bratzler et al., 2013).

Invasive procedures are performed on a daily basis, close to 40 million episodes reported between 2009-2014 in the UK (Abbott et al., 2017). We reason that EPS could be used prior to an invasive procedure, to prime the patient's immune system against potential systemic *S. aureus* infection and improve outcomes. As it will be discussed later, this approach with EPS may have some limitations given that we have not tested EPS against other potential bloodstream pathogens; the protective mechanism of EPS on *S. aureus* infection may not work, or even be detrimental, in infections by other causes. Once again, a better understanding of the mechanisms underlying EPS protection from *S. aureus*, and potentially from other etiologic agents, will help develop EPS into specific therapies that ultimately benefit humanity.

### **EPS Protection from *S. aureus* Bloodstream Infection: Role of IFN- $\gamma$**

#### **The Duality of IFN- $\gamma$ : Initial Observations**

Systemic infection is a complex challenge for the host. The host must mount aggressive pro-inflammatory immune responses to remove the invading pathogen, but in doing so, limit overt inflammation. As we discussed previously, *S. aureus* harbors numerous mechanisms to subvert host immunity, rendering the pro-inflammatory responses ineffective. This persistence inflammation damages the surrounding tissue and impedes organ functions, causing severe conditions such as sepsis.

We initiated this study under the premise that EPS, through its anti-inflammatory properties, could reduce inflammation during *S. aureus* infection to improve disease outcomes. However, we found that the effects of EPS are more complex, inducing hybrid MΦs that perform both pro- and anti-inflammatory functions. In addition, EPS prevented the induction of IFN- $\gamma$  during disease, a paradoxical result given the canonical view of IFN- $\gamma$  as a cytokine that enhances antimicrobial functions of phagocytes (Held et al., 1999). Consistent with our observation, some studies demonstrated that in *S. aureus* bloodstream infection, IFN- $\gamma$  plays a detrimental role for the host since neutralization of IFN- $\gamma$  using antibodies or the infection of IFN- $\gamma$ -deficient mice led to improved survival and reduced bacterial load (Nakane et al., 1995; Sasaki et al., 2000). EPS-treated mice had reduced IFN- $\gamma$  levels both in the serum and in organs after infection with *S. aureus*, leading us to conclude that by limiting IFN- $\gamma$  production, EPS reduces the detrimental effects of IFN- $\gamma$  during pathogenesis, reducing disease burden. However, the specific reason as to how the reduction in IFN- $\gamma$  levels correlates with improved disease outcomes in systemic *S. aureus* infection is not apparent.

Since its discovery, IFN- $\gamma$  has been characterized as a molecule driving immune response against intracellular pathogens (Wheelock, 1965). IFN- $\gamma$  mediates its effects through its receptor, which is a heterodimer of IFN- $\gamma$  receptor 1 (IFN- $\gamma$ R1) and IFN- $\gamma$ R2 (Basu et al., 1988; Hemmi et al., 1989; 1994; Soh et al., 1994). The activation of the IFN- $\gamma$ R activates the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway that stimulates the expression of various genes involved in immune responses (Igarashi et al., 1994; Sakatsume et al., 1995). In spite of the studies showing that the absence of IFN- $\gamma$  led to improved survival and reduced bacterial load (Nakane

et al., 1995; Sasaki et al., 2000), the general consensus is that IFN- $\gamma$  plays a protective role during *S. aureus* infection. This is because the treatment of M $\Phi$ s with IFN- $\gamma$  *in vitro* augments their activation (Held et al., 1999; Murray et al., 1985), and treatment of neutrophils with IFN- $\gamma$  *in vitro* enhances their capacity to kill *S. aureus* (Edwards et al., 1988). *In vivo*, immunization of mice through primary infection with a low inoculum of *S. aureus* protects mice from subsequent re-challenge with *S. aureus* 8 weeks later, and this protection is abrogated in IFN- $\gamma$ -deficient mice (Sasaki et al., 2006), suggesting that IFN- $\gamma$  mediates protection *in vivo*. In addition, systemic administration of recombinant IFN- $\gamma$  to mice reduces mortality during *S. aureus* bloodstream infection (Zhao et al., 1998), consistent with the consensus view that IFN- $\gamma$  plays a protective role.

However, a closer look into the studies of *S. aureus* infection involving IFN- $\gamma$  reveals a more complicated story. Zhao and colleagues initially reported that the administration of recombinant IFN- $\gamma$  protected mice from *S. aureus* bloodstream infection (Zhao et al., 1998). In contrast, this group previously reported that IFN- $\gamma$ R-deficient mice have increased arthritis severity during systemic infection by *S. aureus*, but also noted that at later points of infection, fewer IFN- $\gamma$ R-deficient mice succumbed to disease compared to WT mice, suggesting a pathological role of IFN- $\gamma$  signaling (Zhao and Tarkowski, 1995). These results were corroborated by another group that administered neutralizing anti-IFN- $\gamma$  antibodies to C57Bl/6 mice during infection and found improved survival (Nakane et al., 1995). Also, Sasaki and colleagues used IFN- $\gamma$ -deficient mice and showed improved survival and reduced bacterial load during *S. aureus* bloodstream infection (Sasaki et al., 2000), providing a view that contradicts the general view that IFN- $\gamma$  is protective.

## **The Duality of IFN- $\gamma$ : Role of Neutrophils**

There is an apparent paradox regarding the protective and pathogenic roles of IFN- $\gamma$  during *S. aureus* bloodstream infection. Although the underlying reason for this duality of IFN- $\gamma$  remains unclear, one hypothesis to explain this can be derived from a critical observation made in IFN- $\gamma$ -deficient mice. When Sasaki and colleagues assessed the kidney of infected IFN- $\gamma$ -deficient mice for histopathology, they noticed smaller and fewer abscesses, with neutrophils present but the borders unclear (Sasaki et al., 2000). Usually, abscess from *S. aureus* infections show a necrotic focus containing viable bacteria with neutrophils surrounded by a fibrous capsule. Under the premise that abscess is a host mechanism to contain the bacterial infection, such finding of an abscess with unclear borders would implicate incomplete control of bacterial dissemination. However, this is not the case, since these mice had improved survival. In addition, abscess formation is also promoted by the bacteria as well, since bacterial factors from *S. aureus* are required (Cheng et al., 2009; Lam et al., 1963). This suggests that *S. aureus* might utilize the abscess as a way to promote its own pathogenic program.

One target of *S. aureus* manipulation of host response is the neutrophil. The infected site becomes concentrated with neutrophils as they are recruited to clear the pathogen. These neutrophils augment their antimicrobial functions through NETosis (Brinkmann et al., 2004). Ironically, NETosis has been implicated in *S. aureus* immune evasion. Thammavongsa and colleagues assessed renal abscesses from *S. aureus*-infected mice using immunohistochemical staining and found that the inner parts of the abscess only contained Ly6G<sup>+</sup> neutrophils. In contrast, F4/80<sup>+</sup> M $\Phi$ s were concentrated around the periphery of the abscess, excluded from the central infected foci (Thammavongsa et al., 2013). These investigators also observed that in mice infected

with mutants deficient in nuclease (Nuc) and adenosine synthase A (AdsA), F4/80<sup>+</sup> cells were found within the abscess, indicating that this mutant *S. aureus* failed to exclude MΦs from the abscess. This was mediated by *S. aureus* converting host DNA released from neutrophils during NETosis into deoxyadenosine, known to be toxic to MΦs (Thammavongsa et al., 2013). Not surprisingly, infection with one of these mutants, *adsA*, resulted in reduced CFU *S. aureus* in the kidney (Thammavongsa et al., 2009). It should be noted that NETosis is probably not required for *S. aureus* to obtain DNA from neutrophils since *S. aureus* can also use the pore-forming toxin LukED to directly kill neutrophils (Alonzo III et al., 2012a) and release DNA from neutrophils. Therefore, it can be inferred that while neutrophils play a critical role in containing the initial infection by *S. aureus*, they also serve as a pool of materials that *S. aureus* have adapted to utilize for immune evasion.

This view that the neutrophils are utilized by *S. aureus* during pathogenesis is supported by the studies assessing the role of neutrophils in systemic *S. aureus* infection. The initial studies utilized *in vivo* depletion of neutrophils using anti-Gr-1 antibodies that demonstrated that 10 of 13 neutrophil-depleted mice had died from bloodstream infection by *S. aureus* by 2 d.p.i. compared to 0 of 14 deaths in control mice (Verdrengh and Tarkowski, 1997). It should be noted here that the extreme sensitivity of neutrophil-depleted mice in the study by Verdrengh and Tarkowski may be due to the fact the authors likely depleted both monocytes and neutrophils since anti-Gr-1 antibody (RB6-8C5) targets both Ly6C<sup>+</sup> monocytes and Ly6G<sup>+</sup> neutrophils. Still, another study has used anti-Ly6G antibody (1A8) to specifically deplete neutrophils *in vivo*, which also rendered mice more prone to death during *S. aureus* bloodstream infection (Alonzo III et al., 2012a). The presumed protective role of neutrophils in *S. aureus* infection came

into question when investigators unexpectedly observed that an influx of neutrophils correlated with increased CFU *S. aureus* (Lowrance et al., 1994). In a study using an anti-Gr-1 antibody to limit, but not deplete, neutrophils *in vivo*, Gresham and colleagues found a decrease in bacterial burden during systemic *S. aureus* infection (Gresham et al., 2000). Combined with the observation that *S. aureus* readily survives within leukocytes (Rogers and Tompsett, 1952), these data indicate that neutrophils, while required for host survival during *S. aureus* disease, also serve as a niche for *S. aureus* survival.

### **The Duality of IFN- $\gamma$ : Linking IFN- $\gamma$ with Neutrophils**

The observation linking the alteration of abscesses in IFN- $\gamma$ -deficient mice and neutrophils came from a study in which McLoughlin and colleagues observed that TCR $\alpha$ / $\beta$ -deficient mice had reduced numbers of *S. aureus* within abscesses and reduced local levels of CXC motif chemokines such as KC (CXCL1) and MIP-2 (CXCL8), which correlated with reduced myeloperoxidase (MPO) activity in tissues (McLoughlin et al., 2006). They also observed that IFN- $\gamma$ -deficient mice had slightly reduced levels of KC at 6 h.p.i. and MIP-2 at 48 h.p.i., with reduced MPO activity (McLoughlin et al., 2008), suggesting that IFN- $\gamma$  promotes neutrophil recruitment through local production of CXC motif chemokines, leading to increased bacterial burden within the abscess. Similarly, the study of *sea* mutant demonstrated that *sea*-infected mice had reduced levels of IFN- $\gamma$  in the liver, which correlated with a reduced number of hepatic abscesses (Xu et al., 2014). This study also reported that the number of CD11b<sup>+</sup>Ly6G<sup>+</sup> neutrophils was reduced in the liver by 96 h.p.i., consistent with the view that IFN- $\gamma$  promotes neutrophil recruitment (Xu et al., 2014). These data indicate that IFN- $\gamma$  contributes to recruitment of neutrophils during *S. aureus* infection, thereby increasing the cells *S. aureus* can target for its own survival within the infected site.

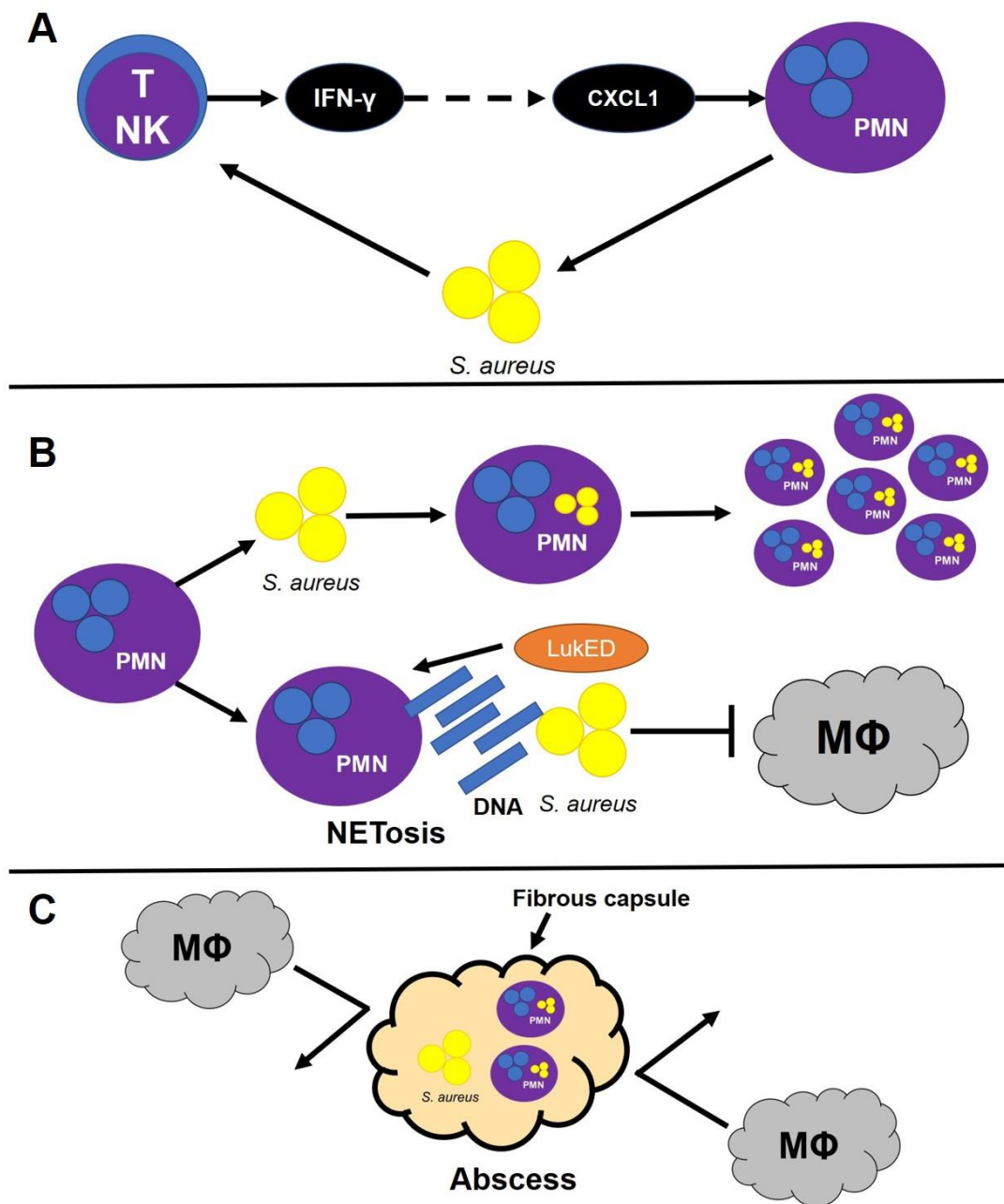


## **The Duality of IFN- $\gamma$ : Role of Macrophages**

While the studies of neutrophils reveal the nature of *S. aureus*-induced abscess as a potential survival mechanism, they only reported a reduction in the number and size of abscesses during infection. As yet, there are no data to directly explain how in IFN- $\gamma$ -deficient mice, the abscess structure was less organized, and the borders obscured (Sasaki et al., 2000). The periphery of the abscess is described by a fibrous capsule that surrounds the necrotic region (Kobayashi et al., 2011), and the excluded M $\Phi$  are located just outside the periphery of the abscess (Thammavongsa et al., 2013). M $\Phi$ s are known to express a variety of matrix metalloproteinases (MMP) upon both classical and alternative activation (Huang et al., 2012), and these MMPs mediate breakdown of extracellular matrix components (Gross and Lapiere, 1962; Wart and Birkedal-Hansen, 1990). We suggest that in IFN- $\gamma$ -deficient mice, the reduction in neutrophil numbers in the abscess leads to reduced *S. aureus*-induced toxicity against M $\Phi$ s, thereby increasing M $\Phi$  presence around the abscess. These M $\Phi$ s could utilize their MMPs to break down the fibrous capsule surrounding the abscess to gain access to the abscess; indeed, a corresponding increase in F4/80<sup>+</sup> cells within abscesses can be observed when *S. aureus* cannot produce deoxyadenosine to exclude M $\Phi$ s (Thammavongsa et al., 2013). This MMP-mediated breakdown of the abscess border may explain why the abscess borders are obscure in IFN- $\gamma$ -deficient mice, and may also signify that in these mice, the abscess is more accessible by immune cells, further enhancing host responses to *S. aureus*. Assessing the status of extracellular matrix components in IFN- $\gamma$ -deficient mice during *S. aureus* infection could reveal if the breakdown in the fibrous capsule is responsible for the obscure borders.

### **Proposed Model of the Role of IFN- $\gamma$ During *S. aureus* Pathogenesis**

In conclusion, abscess formation, and the role of IFN- $\gamma$  during *S. aureus* infection are complex processes that require further studies to elucidate. At present, we propose the following model (**Figure 37**) for the role of IFN- $\gamma$  in *S. aureus* pathogenesis. *S. aureus* induces an early IFN- $\gamma$  response from T or NK cells that leads to the local production of CXC motif chemokines such as KC (CXCL1) in the tissues, which promotes neutrophil recruitment and initiates abscess formation (**Figure 37A**). The source of CXCL1 is not known (**Figure 37A**). Within the abscess, PMNs take up *S. aureus* for killing, but *S. aureus* survives within PMNs. The more PMNs take up the bacteria, the more *S. aureus* survives within the PMNs, increasing the CFU *S. aureus* within the abscess (**Figure 37B**). PMNs augment their antimicrobial functions through NETosis, which releases chromatin to trap the invading bacteria, but *S. aureus* utilizes this DNA for its own survival, killing M $\Phi$ s that are additionally recruited (**Figure 37B**). *S. aureus* also uses leukotoxins to kill neutrophils, releasing more DNA to kill M $\Phi$ s (**Figure 37B**).



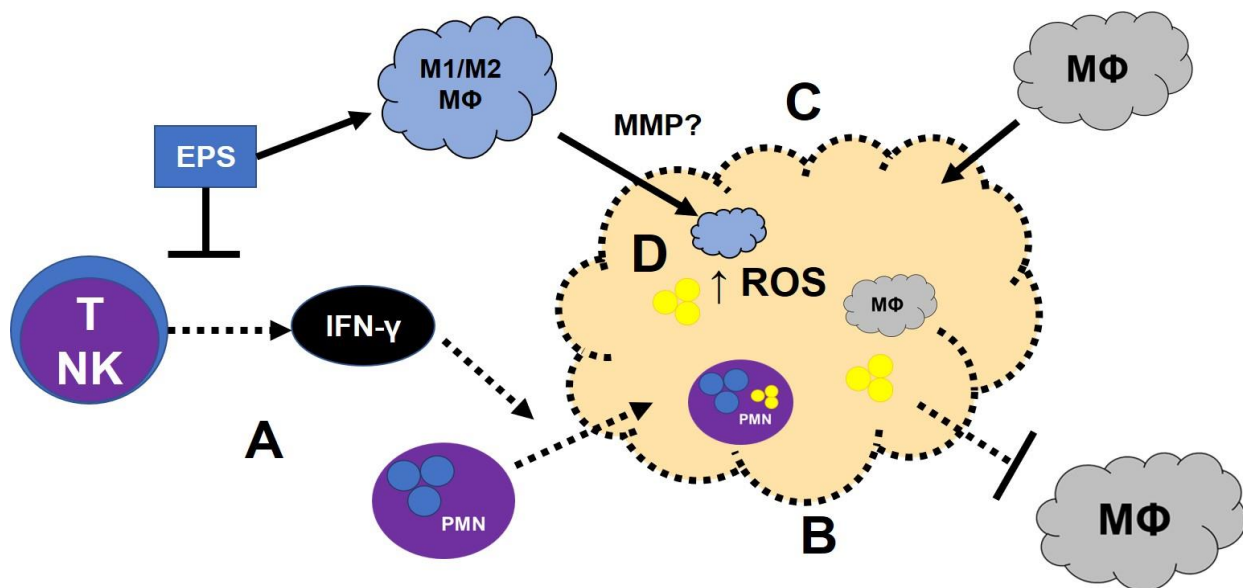
**Figure 37: Proposed Model for the Role of IFN- $\gamma$  and Abscess Formation During *S. aureus* Pathogenesis.** **A.** *S. aureus* stimulates IFN- $\gamma$  production which promotes PMN recruitment through chemokine production. Dashed arrow indicates that the source of CXCL1 is not known. **B.** *S. aureus* readily survives within PMNs after phagocytosis. *S. aureus* utilizes DNA from PMNs to evade MΦ-mediated immunity. **C.** *S. aureus* excludes MΦs from the abscess outside the fibrous capsule, creating an immune-privileged site for bacterial persistence.

These processes, stemming from the initial IFN- $\gamma$  production, form an abscess that

restricts access by immune cells, allowing *S. aureus* an immune-privileged environment to persist within the host (**Figure 37C**).

### Proposed Effect of EPS Suppression of IFN- $\gamma$ on *S. aureus* Infection

We demonstrated that EPS prevents the production of IFN- $\gamma$  during *S. aureus* infection *in vivo*. Based on the proposed role of IFN- $\gamma$  in *S. aureus* pathogenesis (**Figure 37**), we suggest that EPS disrupts the formation of abscesses, thereby reducing bacterial load (**Figure 38**). With the reduction in IFN- $\gamma$  levels, the recruitment of neutrophils to the site of infection would be disrupted, and there would be a corresponding decrease in CFU *S. aureus* persisting within neutrophils (**Figure 38A**).



**Figure 38: Proposed Model of the Effect of EPS on *S. aureus* within Abscesses.** **A.** EPS inhibits IFN- $\gamma$  during *S. aureus* infection, disrupting neutrophil-mediated abscess formation. **B.** Reduced neutrophil recruitment to abscesses reduces MΦ toxicity and subsequent exclusion by *S. aureus*. **C.** EPS-induced hybrid MΦs penetrate abscesses through MMPs, disrupting the abscess capsule to render it more accessible by other MΦs. **D.** EPS-induced hybrid MΦs restrict *S. aureus* growth within the abscess through increased ROS levels. Dashed arrows indicate disruption of process by EPS. Yellow circles represent *S. aureus*.

Furthermore, there would be a decrease in the amount of deoxyadenosine produced by *S. aureus* using neutrophil DNA due to a reduction in neutrophil recruitment, which would reduce toxicity against MΦs (**Figure 38B**). This, in turn, could increase MΦ access into the abscess where bacteria are present, reducing *S. aureus* numbers (**Figure 38B**). Since activated MΦs express MMPs (Huang et al., 2012), EPS-activated MΦs may also have increased MMP expression to penetrate the abscess better (**Figure 38C**). We also demonstrated that EPS-induced MΦs had increased antimicrobial activity through ROS production (**Figure 13-15**), which would contribute to better pathogen control once the cell takes up *S. aureus* within the abscess (**Figure 38D**). Visualization of the abscesses in EPS-treated mice during *S. aureus* infection, particularly the periphery of the abscesses and the surrounding extracellular matrix components, will help test the validity of this model.

### **How IFN-γ Can Contribute to Protection from *S. aureus***

There is one key observation we made that creates a discrepancy for the proposed model for EPS-mediated protection from *S. aureus* infection. We proposed that the reduction in IFN-γ levels in EPS-treated mice disrupted the recruitment of neutrophils to the abscess, which would then correlate with reduced CFU *S. aureus* (**Figure 38A**). However, we observed that EPS recruits neutrophils to tissues (Paynich, unpublished data and **Figure 35**). One explanation for this apparent discrepancy can be found in yet another discrepancy, the finding that mice treated with recombinant IFN-γ are protected from *S. aureus* bloodstream infection (Zhao et al., 1998). In this case, recombinant IFN-γ was administered to mice systemically (Zhao et al., 1998), which would theoretically activate all cells expressing the IFN-γR. While *S. aureus* survives within neutrophils, and the literature suggests that neutrophils contribute to *S.*

*aureus* persistence, IFN- $\gamma$ -activated neutrophils readily kill *S. aureus* (Edwards et al., 1988). Enhancing the bactericidal capacity of individual neutrophils *in vivo*, e.g., by systemic administration of recombinant IFN- $\gamma$  (Zhao et al., 1998), should reduce the initial burden of *S. aureus* at the infected sites, leading to improved outcomes. We observed that EPS activates BM-PMNs (**Figure 36**), so EPS could systemically enhance their antimicrobial activities against *S. aureus in vivo*, which would lead to the better killing of *S. aureus* by PMNs, thereby reducing overall bacterial burden independent of the effects of EPS on IFN- $\gamma$  and abscess formation.

### **The Source of IFN- $\gamma$ During *S. aureus* Infection *In Vivo***

Perhaps reflective of the important role IFN- $\gamma$  plays during *S. aureus* pathogenesis, *S. aureus* harbors two classical and two alternative mechanisms to induce host IFN- $\gamma$  production. The two classical mechanisms are Ag-mediated activation of cognate T cells and SAg-mediated polyclonal activation of T cells. In both cases, T cells are activated and undergo proliferation and produce effector cytokines. Experimentally, the activation of mouse T cells with CP8, a capsular polysaccharide Ag of *S. aureus*, resulted in IFN- $\gamma$  production 72 hr post stimulation (McLoughlin et al., 2008). In our studies, high levels of IFN- $\gamma$  were detected 96 hr after stimulation with SEI-Q containing culture supernatants (**Figure 26B**). However, the *in vitro* kinetics of IFN- $\gamma$  do not match the kinetics of IFN- $\gamma$  levels during *S. aureus* bloodstream infection *in vivo*. Nakane and colleagues reported that during systemic *S. aureus* infection, serum IFN- $\gamma$  was detectable at low levels at 1 d.p.i., undetectable in the following couple days, and then peaked at much higher levels by 5 d.p.i (Nakane et al., 1995). We also detected IFN- $\gamma$  in the serum at 1 d.p.i. (**Figure 4**), but IFN- $\gamma$  was not detectable at 3 d.p.i. (**data not shown**), confirming that IFN- $\gamma$  is produced in a biphasic manner during *S. aureus*

bloodstream infection: an early low-level response at 1 d.p.i. and a surge in levels later in infection, between 3 and 5 d.p.i. SAg-induced IFN- $\gamma$  *in vitro* was detected at 96 hr post stimulation (**Figure 26A and B**), more in line with the timing of the *in vivo* surge later in infection. In contrast, the level of IFN- $\gamma$  at 24 hr after stimulation with SAg was relatively low (**Figure 26A**), so it is not likely that the activation of T cells through the two classic mechanisms is responsible for the early IFN- $\gamma$  response observed at 1 d.p.i. *in vivo*.

Mice infected with the SEA-deficient mutant, compared to WT, reportedly had lower IFN- $\gamma$  levels during infection (Xu et al., 2014), but we did not observe this with our SEL-Q-deficient mutant (**Figure 24**). One possible explanation for this is that because each SAg has specificities for a specific TCR V $\beta$  sequence, the activation profile of the SEA and SEL-Q would be different. Therefore, SEA could activate resident cells in the liver that bear the TCR V $\beta$  that SEA recognizes, while SEL-Q would not. Xu and colleagues also observed that in the Newman strain, *sea* is encoded in the same immune evasion cluster of  $\beta$ -hemolysin converting phage that encodes other immune evasion molecules, especially those involved in evading neutrophil-mediated immunity (Xu et al., 2014). This suggests that *sea* may be co-regulated in a way that promotes expression during neutrophil influx early in the disease. In contrast, the *seq* gene is only neighbored by the *sek* gene, so SEL-Q may not be expressed in the manner *sea* is expressed *in vivo*. Still, the decrease in IFN- $\gamma$  level in the *sea*-infected mice was only partial (Xu et al., 2014), consistent with the view that *S. aureus* can induce IFN- $\gamma$  through other means.

*S. aureus* has alternative mechanisms to induce IFN- $\gamma$  responses that are more rapid. We found that the stimulation of splenocytes with heat-killed *S. aureus*-induced IFN- $\gamma$  that was in low but detectable levels at 24 hr post stimulation (**Figure 26C**). The

production of IFN- $\gamma$  through this method required NK1.1<sup>+</sup> cells, CD11b<sup>+</sup> cells, MyD88 signaling, and IL-12 (**Figure 27-29**), suggesting that *S. aureus* activates myeloid cell production of IL-12 that activates NK cells for the production of IFN- $\gamma$ . Another pathway may involve a specific subset of T cells. Ag-experienced CD8<sup>+</sup> T cells were demonstrated to produce IFN- $\gamma$  within 6 hr of stimulation with Pam3CSK4 or resiquimod (R-848), TLR1/2 and TLR7 agonists, respectively (Salerno et al., 2016). Since *S. aureus* stimulates immune cells through TLR2, it is possible that these CD8<sup>+</sup> T cells could be activated by *S. aureus* directly through TLR2, leading to rapid production of IFN- $\gamma$ . In our hands, depletion of NK1.1<sup>+</sup> cells (NK and NKT cells) resulted in complete abrogation of IFN- $\gamma$  production, but depletion of CD3<sup>+</sup> cells (T and NKT cells) resulted in only a slight decrease that was not statistically significant (**Figure 27**). Based on these data, we suggest that NK cells are the primary mediators of the early IFN- $\gamma$  response to *S. aureus* infection. We cannot rule out the possibility that NKT cells and T cells, especially certain subsets of CD8<sup>+</sup> T cells, make a minor contribution to the rapid IFN- $\gamma$  response to *S. aureus*, and studies utilizing NK cell-depleted mice would allow us to determine if non-NK cells contribute. However, we favor the idea that the NK cells are the major contributors since in mice infected with *L. monocytogenes*, another Gram positive pathogen, NK1.1<sup>+</sup> cells were the dominant IFN- $\gamma$ -producing compartment at 19 h.p.i., an early time point in infection (Thäle and Kiderlen, 2005).

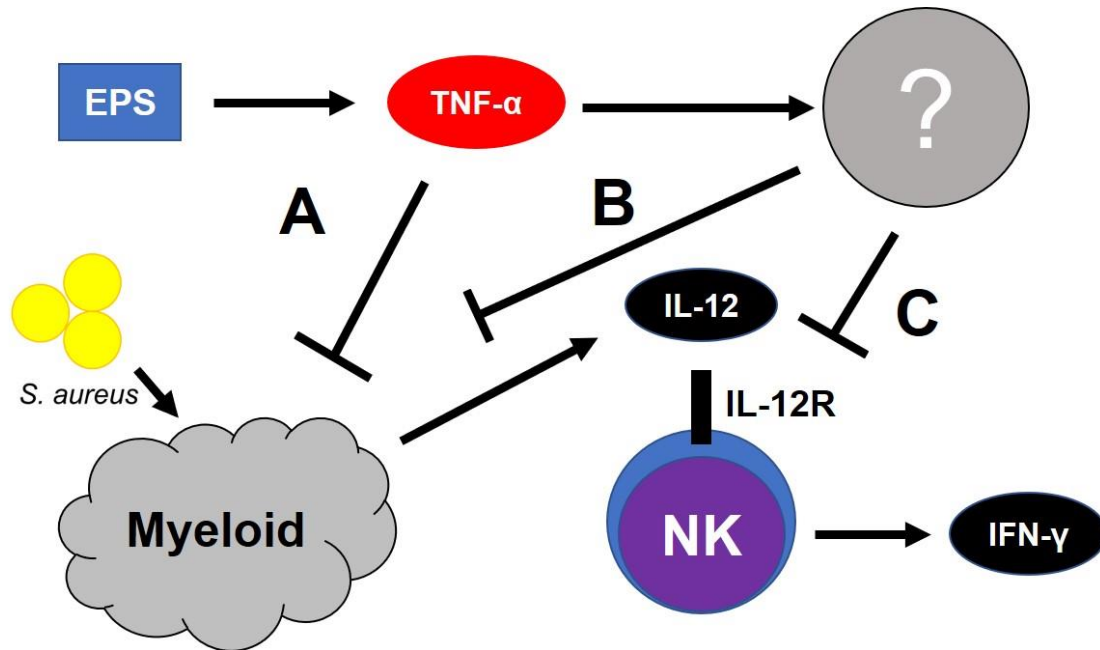
### **EPS Suppression of IFN- $\gamma$ Production Through TNF- $\alpha$**

EPS suppressed splenic IFN- $\gamma$  production in response to heat-killed *S. aureus* (**Figure 30**), and this required TNF- $\alpha$  (**Figure 34**). TNF- $\alpha$  has been classified as a pro-inflammatory cytokine since its discovery as an endotoxin-induced serum factor (Carswell et al., 1975). However, TNF- $\alpha$  has a much more complex set of functions,



mediated through TNF receptor 1 and 2 (TNFR1/2). When activated, TNFR1 recruits an adaptor protein called TNFR type 1-associated death domain protein (TRADD) which mediates two seemingly counteractive signaling pathways: Fas-associated death domain protein (FADD)-mediated caspase activation leading to apoptosis and TNFR-associated factor 2 (TRAF2)-mediated NF- $\kappa$ B and AP-1 activation leading to pro-inflammatory gene expression and cell survival (Hsu et al., 1995; 1996). However, most cells are resistant to apoptosis when stimulated with TNF- $\alpha$  alone (Sugarman et al., 1985), though the reason for this is still not understood. Therefore, the general view is that TNFR1 mediates pro-inflammatory activation and survival of cells. However, a study showed that TNFR1-deficient mice had increased percentages of CD8<sup>+</sup> T cells after immunization (Speiser et al., 1996), indicating that TNFR1 promotes peripheral deletion of cytotoxic T cells. In addition, TNFR1 signaling was demonstrated to induce myeloid-derived suppressor cells (MDSC) that induced T cell anergy during *S. aureus* peritonitis (Ledo et al., 2018), indicating an immunosuppressive role. TNFR2 differs from TNFR1 in that it lacks the death domain. Because of this, TNFR2 was initially thought to only mediate cell survival (Tartaglia et al., 1991). However, a later study showed that TNFR2 could also mediate cell death (Holler et al., 1992), indicating that the signals induced by TNFR2 are also complicated. While TNFR1 is expressed ubiquitously, TNFR2 expression is more limited, generally to the immune system and the nervous system (Ware et al., 1991; Yang et al., 2002). Predominant expression of TNFR2 is found on the maximally suppressive subset of T<sub>reg</sub> cells (Chen et al., 2008), and TNF is thought to increase survival and expansion of this T<sub>reg</sub> cell compartment to promote immunoregulation in response to inflammation (Grinberg-Bleyer et al., 2010). Since EPS induces TNF- $\alpha$  production, we think that some of the immunosuppressive effects of

EPS, especially the increase in T<sub>reg</sub> cells (Paynich et al., 2017) and the reduced response to *S. aureus* SEI-Q (**Figure 19**), may be mediated in part through TNFRs. TNF- $\alpha$  is also crucial for survival during *S. aureus* bloodstream infection (Nakane et al., 1995), suggesting that TNF- $\alpha$ -mediated effects of EPS likely play a role in protection from *S. aureus* infection. Studies using TNFR1- and TNFR2-deficient mice will help us test this hypothesis.



**Figure 39: Proposed Model for the Role of TNF- $\alpha$  in EPS Suppression of NK Cell IFN- $\gamma$  Production.** **A.** EPS-induced TNF- $\alpha$  directly inhibits myeloid IL-12 production in response to *S. aureus*, reducing NK cell activation. **B.** EPS-induced TNF- $\alpha$  indirectly inhibits myeloid production of IL-12 in response to *S. aureus*, through a yet unidentified mediator cell. **C.** EPS-induced IFN- $\gamma$  inhibits NK cell sensitivity to IL-12, likely through a yet unidentified mediator cell. The mediator could consist of multiple cell types and signaling pathways.

TNFR1 and TNFR2 are both expressed on NK cells, but there is no explanation as to why EPS would require TNF- $\alpha$  to suppress IFN- $\gamma$  production by the NK cells. This is because TNF- $\alpha$  was shown to mediate the opposite for NK cells, as the addition of TNF- $\alpha$  or a TNFR2 agonist enhances IFN- $\gamma$  production stimulated by IL-2 and IL-12 in human NK cells (Almishri et al., 2016). Therefore, we suggest that EPS-induced TNF- $\alpha$  does not directly act on the NK cell itself. We think that there are three possible mechanisms by which EPS-induced TNF- $\alpha$  limits IL-12-dependent activation of NK cells in response to *S. aureus*. First, TNF- $\alpha$  could act on the myeloid compartment to limit their IL-12 production in response to *S. aureus* (**Figure 39A**), leading to reduced activation of NK cells and their IFN- $\gamma$  production. While we could not detect differences in IL-12p70 levels in spleen, liver, and kidney of PBS- and EPS-treated mice during *S.*

*aureus* infection (**Figure 21-23**), we did observe reduced serum levels of IL-12p70 in EPS-treated mice at 1 d.p.i. (**Figure 4**), suggesting that EPS has the capacity to limit IL-12 production. Another possibility is that EPS-induced TNF- $\alpha$  acts on a yet unidentified mediator cell, perhaps an MDSC, that then inhibits the production of IL-12 in response to *S. aureus*, leading to reduced NK cell activation (**Figure 39B**). It could also be that this mediator cell act on NK cells to reduce their sensitivity to IL-12, such as downregulating IL-12R levels on NK cells (**Figure 39C**), leading to reduced NK cell activation by IL-12. Identifying the source of IL-12 during *S. aureus* infection *in vivo* that is responsible for the activation of NK cells and assessing the expression of IL-12R on NK cells will help elucidate this mechanism further.

### **EPS Protection from *S. aureus* Bloodstream Infection: Role of Macrophages**

#### **Antimicrobial Mechanisms of Hybrid Macrophages: ROS**

EPS-induced M $\Phi$ s were previously characterized as anti-inflammatory M2 M $\Phi$ s that limit T cell activation (Paynich et al., 2017). Our results showed that EPS-induced M $\Phi$ s are hybrid M $\Phi$ s with both M1- and M2-like qualities, able to restrict *S. aureus* growth through ROS and limit T cell activation by *S. aureus* SEI-Q. In mediating opsonophagocytic killing, M $\Phi$ s use a variety of mechanisms to kill bacteria; the two major mechanisms are RNS and ROS production. While RNS did not explain the enhanced restriction of *S. aureus* by EPS-induced M $\Phi$ s, RNS was produced by these M $\Phi$ s, suggesting that RNS contributes to the restriction of *S. aureus* growth (**Figure 16**). In demonstrating that ROS was responsible for the enhanced restriction, we used DPI, an irreversible inhibitor of NADPH oxidase (Cross and Jones, 1986). However, DPI is known to have off-target effects that lead to inhibition of mtROS production as well (Li and Trush, 1998), so we cannot identify the source of ROS responsible for restriction

of *S. aureus* growth. However, recent studies have highlighted the close relationship between mtROS, M2 MΦs, and killing of *S. aureus*, leading us to think that mtROS play a vital role in the restriction of *S. aureus* growth.

ROS production from the mitochondria is thought to occur as a byproduct of mitochondrial metabolic processes since increasing mitochondrial respiration increases MΦ ROS levels (Arsenijevic et al., 2000). One key feature of M2 MΦ differentiation is fatty acid oxidation (Huang et al., 2014), a mitochondrial metabolic process. Therefore, M2 MΦs are expected to produce higher levels of mtROS, and indeed, EPS-induced MΦs had higher mtROS levels (**Figure 14B**). A recent study showed that during *S. aureus* infection, MΦs recruit mtROS-containing vesicles derived from the mitochondria to the *S. aureus*-containing phagosomes (Abuaita et al., 2018), suggesting that mtROS are involved in the MΦ response to internalized *S. aureus*. The importance of the mitochondria in MΦ-mediated immunity is further highlighted by the discovery that *S. aureus* alpha-toxin drives the mitochondria away from *S. aureus*-containing phagosomes (Cohen et al., 2018), indicating that *S. aureus* evades the mitochondria to promote its survival within the MΦ. While EPS-induced MΦs also increase their mtROS levels upon infection with *S. aureus*, we do not know if EPS-induced MΦs utilize mtROS to restrict *S. aureus* growth. Examination of the mtROS and their recruitment within EPS-induced MΦs will help us determine if EPS-induced MΦs use mtROS for restriction of *S. aureus* growth.

### **Antimicrobial Mechanisms of Hybrid Macrophages: Other Potential Mechanisms**

While ROS was required for restriction of *S. aureus* growth by EPS-induced MΦs, we cannot rule out the possibility that other mechanisms also contribute. One

particular way MΦs can capture intracellular pathogens for killing is through autophagy, but it is not clear if autophagy benefits MΦs in their response to internalized *S. aureus*. In fact, accumulation of autophagosomes was associated with enhanced survival of *S. aureus* in BMDCs (O’Keeffe et al., 2015), suggesting that autophagy may be detrimental for the host. However, this result is in part due to *S. aureus* manipulating the autophagic processes. *S. aureus* is known to activate the mammalian target of rapamycin (mTOR) through to transit into autophagosomes which prevents its maturation to promote persistence (Schnaith et al., 2007). M2 MΦ differentiation involves the activation of the mTOR pathway, which drives cells to rely on mitochondrial metabolism (Huang et al., 2016). Therefore, we hypothesize that EPS-induced MΦs may have an altered state of mTOR activation, which could alter the autophagic response to *S. aureus* infection. In addition, EPS-induced MΦs could promote maturation of the autophagosomes, leading to enhanced restriction of *S. aureus* within the cells. Consistent with this model, TLR4 has been implicated in promoting autophagy through Toll-IL-1R domain-containing adaptor-inducing IFN-β (TRIF) signaling, so we think that EPS could also do this through TLR4-TRIF signaling. We will need further studies utilizing *S. aureus* mutants that cannot induce autophagy or autophagy-deficient MΦs to test if this process is involved in EPS-mediated protection.

### **Induction of Hybrid Macrophages by EPS**

Unlike the effector functions of EPS-induced hybrid MΦs, the mechanisms underlying the induction of these cells by EPS is poorly understood. Paynich and colleagues previously demonstrated that treatment of peritoneal MΦs with EPS *in vitro* results in increased expression of M2 MΦ markers (Paynich et al., 2017), but these MΦs do not recapitulate all of the functions of MΦs isolated from EPS-treated mice (**data**

**not shown**). To understand why these *in vitro*-stimulated MΦs did not function like EPS-induced MΦs *in vivo*, we need to consider the dynamic nature of peritoneal MΦ subsets. The majority of peritoneal cavity MΦs at steady state are CD11b<sup>high</sup>F4/80<sup>high</sup> LPMs (Ghosn et al., 2010). Therefore, *in vitro* experiments assessing the effect of EPS on MΦs were performed on LPMs. However, the peritoneal cavity becomes dominated by CD11b<sup>+</sup>F4/80<sup>+/low</sup> SPMs after EPS treatment *in vivo* (Paynich, unpublished data), and there is a corresponding decrease in the LPM compartment. By 3 days after EPS treatment, the LPM compartment is restored, and the MΦ compartment then consists of functional hybrid MΦs. While we do not know the mechanism underlying the induction of hybrid MΦs *in vivo*, we propose a model that potentially explains the process considering the dynamics of peritoneal cavity MΦs.

### **Ontogeny of Peritoneal Macrophages**

A hint for the origins of EPS-induced hybrid MΦs can be found in the study of peritoneal LPMs and SPMs. Ghosn and colleagues compared the LPMs and SPMs in their ability to produce the antimicrobial molecule, RNS, and found that when isolated 20 hr after i.p. LPS treatment, SPMs increase NO levels higher than that of LPMs; SPMs also had higher levels of MHCII (Ghosn et al., 2010). SPMs were shown to be derived from the infiltrating CD11b<sup>+</sup>Ly6C<sup>+</sup> monocytes (Ghosn et al., 2010), so it is thought that these are cells recruited to mediate antimicrobial responses against invading pathogens. EPS induces the infiltration of CD11b<sup>+</sup>Ly6C<sup>+</sup> monocytes into the peritoneal cavity (Paynich, unpublished data), so we hypothesize that EPS-induced hybrid MΦs may originate from the recruited monocytes like SPMs, which could explain the enhanced antimicrobial capacities of these cells. Consistent with this view is that EPS induces the production of a variety of chemokines by the peritoneal F4/80<sup>+</sup> cells (**Figure 31**), which

would recruit SPMs. It should be noted that in comparing LPMs and SPMs, Ghosn and colleagues did not assess ROS production, so we will need to confirm that SPMs have increased ROS levels like in EPS-induced MΦs. Also, we will need to confirm if EPS-induced MΦs are derived from cells in the circulation using fate mapping or adoptive transfer approaches.

### **Signals Governing EPS-Induced Macrophage Differentiation**

If EPS-induced MΦs originate from the infiltrating monocytes that are classically characterized to mediate pro-inflammatory responses, how is it that these MΦs mediate anti-inflammatory functions as well? We think that the answer lies in the signaling events that occur after recruitment. Paynich and colleagues demonstrated that treatment of peritoneal MΦs increases their expression of IL-4Rα (Paynich et al., 2017), indicating that EPS sensitizes cells to activation by IL-4 or IL-13. While we have not assessed if this also occurs on the recruited SPMs, the increase in IL-4Rα depends on IL-10 (**Figure 33**), a cytokine produced by type I IFNs, resulting from TLR4-TRIF signaling (Chang et al., 2007). Since EPS activates NF-κB through TLR4 (**Figure 32**) and induces cytokine production multiple cell types (**Figure 31 and 36**), we think that EPS could be activating IL-10 production from the recruited SPMs as well, thus increasing their IL-4Rα expression. If not, IL-10 produced by the LPMs within the peritoneal cavity could act on the recruited SPMs to increase their IL-4Rα expression. Signaling through the IL-4R is the classic mechanism for alternative activation of MΦs (Murray et al., 2014), so we think that by increasing IL-4Rα expression on the SPMs, EPS makes the SPMs prone to alternative activation, leading to their immunoregulatory functions.



Paynich and colleagues demonstrated that EPS treatment increases intracellular levels of IL-4 and IL-13 on LPMs (Paynich et al., 2017), but we could not detect secretion of IL-4 and IL-13 after treatment of peritoneal LPMs with EPS *in vitro* (**Figure 31**). In addition, i.p. administration of EPS results in the initial disappearance of the LPMs as the SPMs get recruited (Paynich, unpublished data), leading us to think that a different cell is the source of IL-4 or IL-13. We do not yet know this source, but a number of different cell types within the peritoneal cavity are known to produce IL-4 and IL-13, leading us to think that these cells could be the source by which EPS drives the induction of hybrid MΦs. Amongst these cells, we think that eosinophils, basophils, type 2 innate lymphoid cells (ILC2), and B cells are the likely cells since they are involved in type 2 immune responses defined by IL-4 and IL-13. Interestingly, iNKT cells in the liver were demonstrated to produce IL-4 after a sterile injury to drive M2 differentiation of the recruited monocytes and promote wound healing (Liew et al., 2017), consistent with the view that an accessory cell drives M2 differentiation in EPS-treated mice. It should be noted that we cannot rule out the possibility that signals other than IL-4 and IL-13 drive EPS induction of hybrid MΦs, so we need to consider other signals that could contribute.

Such an alternative signal to consider for hybrid MΦ differentiation by EPS is MCP-1 (CCL2). During murine systemic infection, MCP-1 is produced early and is thought to mediate the recruitment of monocytes through its receptor CCR2 (Kurihara et al., 1997), thereby promoting inflammation. However, during systemic infection, MCP-1 was associated with immunosuppression and increased mortality (Tsuda et al., 2004). Blockade of MCP-1 in MΦs enhanced M1-associated gene expression while it reduced M2-associated genes, suggesting that MCP-1 promotes M2 polarization of MΦs

(Sierra-Filardi et al., 2014). EPS induces MCP-1 production by peritoneal LPMs (**Figure 31**), so MCP-1 could contribute by not only recruiting monocytes to the peritoneal cavity, but also promote their M2 polarization *in vivo*. In addition, EPS-treatment results in abrogated serum MCP-1 levels during *S. aureus* infection (**Figure 4**). This reduction in MCP-1 during infection may promote MΦs to take on antimicrobial capacities rather than fully adopting M2 polarization as in EPS-treated mice, leading to their hybrid state capable of both antimicrobial and immunoregulatory functions. In addition, CCR2-deficient mice show impaired IFN-γ production in response to immunization with *Mycobacterium bovis* (Peters et al., 2000), another parallel observation consistent with the abrogation of both MCP-1 and IFN-γ during *S. aureus* infection by EPS.

### **Proposed Model of the Induction of Hybrid Macrophages by EPS**

Taking the above information into account, we propose the following model for EPS induction of hybrid MΦs in the peritoneal cavity. EPS initially activates TLR4-MyD88 signaling on LPMs, inducing the production of chemokines that lead to recruitment of SPMs that have increased antimicrobial capacities (**Figure 40A**) (Ghosh et al., 2010). EPS also activates TLR4-TRIF signaling, which causes IL-10 production, driving the expression of IL-4Rα on recruited SPMs (**Figure 40B**). A yet unidentified accessory cell provides IL-4 and IL-13, activating the SPMs and promoting their M2 differentiation (**Figure 40C**). Activation through IL-4Rα converts MΦ metabolism to mitochondria-driven metabolism (Huang et al., 2016), and TLR4 signaling also increases mtROS (West et al., 2011), which contributes to M2 MΦ differentiation (Formentini et al., 2017) (**Figure 40C**). Due to the origin and the differentiation pathway of EPS-induced MΦs, we propose that they harbor qualities of both M1 and M2



immunity by altering the expression of co-stimulatory molecules on Ag-presenting cells (Sanchez et al., 2017). Therefore, EPS-induced MΦs could use other mechanisms to inhibit T cells in the presence of *S. aureus*-derived factors due to the pro-inflammatory signals they receive. We will need to confirm that TGF-β and PD-L1 mediate this inhibition or reassess the mechanisms of inhibition to understand inhibition of SAg-mediated activation of T cells by EPS-induced MΦs.

### **Tissue-Specific Protection from *S. aureus* Infection by EPS**

#### **Role of Interferon-Gamma**

EPS-treated mice have reduced bacterial burden in the spleen and liver, but not in the kidney (**Figure 6A and B**). While we do not understand the mechanism behind this, we suggest that it may be due to the differences in immune cells among these tissues. DCs dominate the immune cell population within the kidney (Kaissling and Hir, 1994; Zheng et al., 2006). This is in contrast to the spleen and the liver that harbor many MΦs and lymphocytes. Nakane and colleagues observed that IFN-γ levels in the kidney remain at baseline levels throughout the infection (Nakane et al., 1995), and we observed that renal IFN-γ levels in *S. aureus*-infected mice at 1 d.p.i. are barely detectable (**Figure 23**). Therefore, we think that the inhibitory effect of EPS on T and NK cells, and the resulting IFN-γ, in the kidney is minimal, since the kidney does not harbor so many of these cells. Consistent with this view, *sea*-infected mice had reduced IFN-γ levels in the liver but not in the kidney (Xu et al., 2014), suggesting that IFN-γ differentially contributes to pathogenesis among organs, which could explain the lack of EPS effects in the kidney. It should be noted that IFN-γ-deficient mice had reduced bacterial load in the kidney, and the observation on abscess formation was made from the kidney (Sasaki et al., 2000), suggesting that IFN-γ plays an overall pathogenic role

in the kidney as well. Therefore, we suggest that EPS does not affect kidney pathophysiology.

### **Role of Myeloid Cells**

Another possibility for the failure of EPS to reduce bacterial load in the kidney may have to do with the myeloid compartment. As discussed, DCs are the dominant immune cell in the kidney (Kaissling and Hir, 1994). Renal DCs are thought to rapidly recruit neutrophils upon recognition of infection such as uropathogenic *E. coli* (Tittel et al., 2012). In the case of *S. aureus* infection, this rapid recruitment of neutrophils may promote abscess formation, thereby increasing *S. aureus* survival. In contrast, the liver harbors Kupffer cells, unique tissue resident MΦs, that clear activated neutrophils and cellular debris (Brown et al., 2001). Clearance of neutrophils would reduce abscess formation within the hepatic tissue and not only help clear the bacteria, but also maintain MΦ access to the invading pathogen. Resident MΦs were recently shown to cloak pathogens from recognition by neutrophils, preventing the initiation of abscess formation and subsequent tissue damage (Uderhardt et al., 2019). This process may also be occurring in the liver through Kupffer cells, reducing abscesses. In addition, peritoneal LPMs have been demonstrated to be directly recruited to the liver during sterile injury, where the LPMs mediated uptake of necrotic tissue debris and promoted wound healing (Wang and Kubes, 2016); this may be another way the liver could recruit more MΦs during *S. aureus* infection to clear neutrophils, suppress abscesses, and promote wound healing. The spleen also contains specialized resident MΦs, some of them known to mediate immunity against bloodborne pathogens (Geijtenbeek et al., 2002), and these could also contribute to clearing activated neutrophils and provide additional immunity against *S. aureus*. Additionally, EPS could enhance the

antimicrobial capacities of MΦs in these tissues to further accentuate the difference in responses in these tissues compared to the kidney.

### **Activation of TLR4 by EPS**

#### **Classic and Modern Views on the Effect of Lipopolysaccharide in Immunity**

EPS has broad anti-inflammatory effects that confer host protection against a variety of diseases where inflammation plays a role, including infectious diarrhea (Jones et al., 2014; Paynich et al., 2017), allergic eosinophilia (Swartzendruber et al., 2019), and sepsis. However, we found that EPS also stimulates immune cells through TLR4, leading to the activation of NF-κB and pro-inflammatory cytokine production. The best-characterized ligand for TLR4 is LPS, an inducer of pro-inflammatory responses, and the model causative agent of sepsis (Hoshino et al., 1999). However, a closer look at the literature on LPS indicates that TLR4 can mediate immunoregulatory responses *in vivo*. The first evidence of this was observed when Uchiyama and colleagues found that while LPS administration at the time of immunization enhanced antibody responses, pre-injecting mice with LPS 3 days prior to immunization reduced antibody responses (Uchiyama et al., 1984). Since then, this phenomenon has been studied in the context of LPS-induced endotoxemia model and has been termed sepsis-induced immunosuppression or immunoparalysis. Generally, the primary mechanistic focus from these studies has been the induction of CD11b<sup>+</sup>Gr-1<sup>+</sup> MDSCs *in vivo* (Greifenberg et al., 2009; Wilde et al., 2009). Therefore, the underlying mechanism for how LPS administration results in a suppressed host immunity is poorly understood outside of the induction of MDSCs. Because EPS also activates TLR4, we think that some of the immunoregulatory mechanisms of EPS could also explain sepsis-induced

immunosuppression. This also suggests that the classic view of TLR4 signaling in mediating pro-inflammatory responses need to be re-examined.

The modern view of immunity is that the immune system, like virtually all other physiologic systems in the body, is subject to extensive homeostatic mechanisms. Such an idea has been highlighted in studies of inflammation that claim the need to balance pro- and anti-inflammatory responses. However, these processes need to be recognized as part of the same process. We hypothesize that LPS-induced immunosuppression, and even EPS-primed immunity, demonstrate this phenomenon: an initial pro-inflammatory response activated through TLR4 that results in host countermeasures to limit and resolve inflammation. MDSCs would also fit into this view since MDSCs are known to be induced through inflammatory mediators such as IL-1 $\beta$  (Elkabets et al., 2010), representing a host response to inflammation that limits or resolves inflammation.

Clinically, sepsis-induced immunosuppression is a primary concern, since many patients that survive the initial inflammatory phase are vulnerable to superinfections, presumably due to the immunosuppressed state that ensues later in the disease. Therefore, agents that limit inflammation have not been well-accepted by the field, in part due to concerns of worsening the immunosuppressed state of sepsis patients. However, we suggest that limiting inflammation during the early phase of sepsis can not only improve patient survival, but also reduce the ensuing anti-inflammatory response that occurs in response to the initial inflammation, essentially a reflex response. In addition, priming the host through TLR4, as shown in our EPS-treated mice, bolsters antimicrobial immunity, indicating that the immunoregulatory effect of TLR4 does not impair antimicrobial immunity to *S. aureus*. Therefore, limiting inflammation during

the acute phase of sepsis using a TLR4-based immunotherapeutic agent such as EPS may be a novel strategy to preserve host immunity during the late phase of sepsis. The effect of EPS treatment on preserving host immunity during the late phase of sepsis will need to be examined, but EPS limits activation of T cells by *S. aureus* SAg which normally results in deletion of certain T cells from the repertoire, thereby providing some evidence for EPS preserving host immunity.

### **EPS and LPS**

EPS and LPS both activate signaling through TLR4. However, LPS is produced as a part of the gram negative cell wall while EPS is produced by the gram positive *B. subtilis*, as a part of its biofilm matrix. Therefore, the structure of EPS, while not known, is expected to be quite dissimilar compared to LPS. While the idea of two dissimilar molecules acting through the same receptor seems unlikely, TLR4 is known to mediate signaling by a vast array of ligands, many of them endogenous ligands such as fibrinogen (Smiley et al., 2001), hyaluronan (Jiang et al., 2005), and S100 proteins (Vogl et al., 2007). The diversity of ligands for TLR4 is possible because TLR4 often works in conjunction with co-receptors, such as CD14 and MD-2 that mediate LPS recognition. Therefore, we hypothesize that EPS has a distinct receptor that acts as a co-receptor on TLR4 to initiate EPS-induced TLR4 signaling, a potential difference from LPS which uses LPS-binding protein, CD14, and MD-2 (Ryu et al., 2017). While the signal itself induced through TLR4 is very similar between EPS and LPS, there may be some differences if a different co-receptor is used because this can affect the downstream adaptors that are recruited or the amount of TLR4 that is endocytosed to mediate TLR4-TRIF signaling. These differences may lead to EPS being used in potentially different therapeutic scenarios than other TLR4-targeting agents. The



elucidation of EPS structure, and the identification of the EPS receptor, will help us understand the biology of EPS-induced signaling, which may even reveal a novel aspect of TLR4 biology.

### **Other Cell Types that Could Mediate Protection by EPS in *S. aureus***

#### **Infection**

Given that EPS activates TLR4 signaling on both MΦs and neutrophils, we hypothesize that many other cell types contribute to EPS-mediated protection from sepsis, given that they express the yet unidentified EPS receptor. The following are a short, incomplete list of cell types that could be affected by EPS during *S. aureus* bloodstream infection.

**Basophils:** Basophils have been demonstrated to protect mice from sepsis induced by cecal ligation and puncture through TNF, and these cells express TLR4 (Piliponsky et al., 2019). TNF- $\alpha$  is crucial for survival during *S. aureus* infection (Nakane et al., 1995), and we hypothesize that EPS could potentially induce TNF- $\alpha$  production by basophils, thereby contributing to protection from sepsis.

**Eosinophils:** Eosinophils have recently been implicated in protection from *S. aureus* sepsis, through a mechanism that is poorly understood (Krishack et al., 2019). We have some evidence that EPS increases the percentage of eosinophils within tissues (Paynich, Fleming-Trujillo, unpublished data), and we suggest that eosinophils could mediate EPS protection from sepsis.

**Endothelial cells:** The major pathologic feature of sepsis, cardiovascular collapse, is thought to initiate from endothelial dysfunction. Endothelial cells were demonstrated to be activated by LPS through TLR4, but this does not lead to endothelial dysfunction as presumed previously (Menghini et al., 2014). Instead, activation of endothelial cells

leads to protection from sepsis, presumably from efficient recruitment of neutrophils to the site of infection (Andonegui et al., 2009). We think that EPS could also activate endothelial cells through TLR4, thereby contributing to protection from sepsis.

**Platelets:** Platelets have been demonstrated to mediate host defense against *S. aureus*. One mechanism that bears uncanny similarity to EPS is the ability to enhance macrophage uptake and restriction of *S. aureus* growth, mediated through IL-1 $\beta$  production by platelets (Ali et al., 2017). Platelets express TLR4 (Andonegui et al., 2005), and stimulation of platelets with LPS leads to the production of IL-1 $\beta$  (Brown et al., 2013). Therefore, we think that EPS could enhance antimicrobial activities of M $\Phi$ s through TLR4-mediated activation of platelets. In addition, activated platelets have been shown to directly kill *S. aureus* by releasing a yet unidentified antimicrobial product (Ali et al., 2017), which could also be a way EPS could promote antimicrobial immunity against *S. aureus*.

### Concluding statement

Sepsis is one of the most dramatic manifestations of infection. Patients are treated immediately and aggressively, but their conditions can rapidly deteriorate, leading to high mortality rates. *S. aureus* is the leading cause of sepsis, and it is incredibly challenging to treat, given its broad antimicrobial resistance profile and an uncanny ability to subvert the immune system to persist and even promote its own survival. Therefore, novel agents and strategies to combat systemic *S. aureus* infection are critically needed. Probiotics represent a vast potential for developing new therapies, but the mechanisms by which they benefit hosts are not known.

In this study, we demonstrated that EPS derived from the probiotic, *B. subtilis*, can protect hosts from *S. aureus* bloodstream infection. EPS does this by priming host

immunity through TLR4, which improves antimicrobial functions of MΦs, and limits inflammation induced during infection. This EPS-primed immunity prevents *S. aureus* from optimally subverting the host processes for its own pathogenic program, leading to reduced disease burden and improved host survival. By priming high-risk patients with EPS, we suggest that we can benefit patients undergoing invasive procedures by limiting severity during subsequent infection that occurs in high frequency. Still, EPS has the potential to affect many other aspects of host physiology beyond the few cell types we presented here, so there could be other scenarios in which EPS could be beneficial. By increasing our understanding of the structure, host receptor, and the molecular pathways of EPS, we will be able to bring EPS, and more broadly TLR4-targeting therapy, to the healthcare industry. In addition, we will be able to open the door to microbial therapy, a novel sector of therapeutics with a vast potential to benefit humanity.

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## VITA

Wonbeom “Won” Paik was born in 1989 and raised in Seoul, Republic of Korea. In 2000, he came to the United States where he spent his time in Chicago and in Aurora, Illinois. He went on to DePaul University for his Bachelor of Science degree in biotechnology with minor in sociology in 2011. During his undergraduate studies, he was introduced to research by studying chromosomal congression in female *Drosophila melanogaster* during meiosis I in the lab of Dr. William Gilliland. In 2011, Won joined the master’s program in Infectious Disease and Immunology at Loyola where he joined the laboratory of Dr. Katherine L. Knight, where he studied vaccine candidates for *Clostridium difficile* infection with the guidance of Dr. Knight and Dr. Dale N. Gerding. Won then received his Master of Science degree in 2013.

Won matriculated into the MD/PhD program at Loyola in 2013 and continued his studies in Dr. Knight’s laboratory on a different topic. He studied the mechanism of protection from systemic *Staphylococcus aureus* infection by *Bacillus subtilis*-derived exopolysaccharide, with the guidance of Dr. Knight and Dr. Francis Alonzo III. During his time in the laboratory, Won was supported by the NIH T32 training grant to Dr. Katherine L. Knight and by the AHA predoctoral fellowship. After completion of his PhD studies, Won will be joining the medical school at Stritch School of Medicine in Maywood, Illinois.



